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Polarization of the Fluorescence of Macromolecules

1. THEORY AND EXPERIMENTAL METHOD

By G. WEBER*
Biochemical Laboratory, University of Cambridge

(*Received* 2 *August* 1951)

According to the theory developed by Perrin (1926), the partial polarization of the fluorescent light emitted by molecules in solution depends upon the relation of their rotational relaxation time to the lifetime of the excited state of the fluorescence.

The measurements of Gaviola (1927), Szymanowski (1935) and Dushinsky (1936) show that the lifetime of the excited state of the fluorescence is a quantity of the order of 10^{-8} sec. for many dyes in water solution. As the relaxation time of the rotation of these molecules in water is much shorter than 10^{-8} sec., almost completely depolarized radiation is to be expected and is in fact observed. On the other hand, fluorescent macromolecules, e.g. proteins, should already emit partially polarized fluorescence in water solution as their relaxation times are of the required order of magnitude. Measurements of the degree of polarization of the fluorescence should in consequence afford a convenient means of determining the relaxation times of macromolecules in dilute solution under a variety of circumstances.

In this paper are described the general principles which attend the application of this method to the study of macromolecules. The following paper of this series (Weber, 1952) describes experiments done with proteins rendered fluorescent by the conjugation with a small fluorescent molecule.

THEORY

Perrin (1926, 1936) has developed the theory of the polarization of the fluorescence of solutions on the assumption that the emitting molecules carry rigidly bound linear oscillators of absorption and emission and that the molecular rotations are described by Einstein's (1906) equation. The first assumption can be considered experimentally proved (Feofilov, 1943). Although the classical experiments of J. Perrin (1909) have shown that the rotation of large, visible particles follows the Einstein equation, some doubt may be entertained as to its validity in the case of dissolved molecules which do not greatly differ in size from the molecules of the solvent. The protein molecules fall midway between these two extremes, and the measurements of their relaxation times by the dielectric dispersion method (Oncley, 1942) indicate that the equation of Einstein cannot seriously be at fault. However, the fact that the real shape of the particles is not known and that the measurements have been carried out over a limited range of temperatures and viscosities does not allow a decision as to how closely the theory is followed.

Let us consider a system of co-ordinates $O(xyz)$. The fluorescent solution is placed at O in a square cell with faces oriented normally to the co-ordinate axes. The exciting light travels in the xO direction, and unless otherwise stated we shall assume it to be completely polarized with its electric vector in the Oz direction. The fluorescent light emitted in the Oy direction, i.e. at right angles both to the direction of propagation of the exciting light and to the direction of the electric vector is observed. Its partial polarization p is defined by the equation

$$p=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+I_{\perp}}, \qquad (1)$$

where $I_{\parallel}$ is the component of the intensity emitted in the direction Oy with its direction of vibration parallel to that of the exciting light, while $I_{\perp}$ is the component normal to the former, i.e. in the Ox direction.

According to Perrin (1926) the value of p for a spherical molecule is given by

$$\frac{1}{p}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left(1+\frac{RT}{\eta V}\tau_0\right), \qquad (2)$$

where R is the gas constant, T the absolute temperature, η the viscosity of the solvent, V the molecular volume of the fluorescent molecule and τ_0 the lifetime of the excited state of the fluorescence; p_0 is clearly the value of p when $T/\eta \to 0$, i.e. when no depolarization by molecular rotation takes place. In practice it differs little from the polarization observed in a medium of high viscosity like glycerol. According to the above equation, if $1/p$ is plotted against T/η a straight line is obtained cutting the $1/p$ axis at $1/p_0$. Experimentally, for fluorescent dyes with $V \simeq 500$ in glycerol-water mixtures it is found that the relation just mentioned obtains down to viscosities of 10–15 centipoises, but for

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viscosities below these values the polarization is lower than predicted (Perrin, 1926; Wawilow, 1936). Several explanations have been advanced, notably that Einstein's law is not valid in these conditions (Perrin, 1936). Whatever the explanation, if a change in molecular volume has the same effect as a corresponding change in viscosity, Perrin's law should hold accurately for molecules of $V > 10^4$ in media of viscosity of 1 centipoise.

Simultaneous excitation of several oscillators

If several oscillators corresponding to one or more molecular species in solution are simultaneously excited, the observed polarization $\bar{p}$ is related to the individual polarizations p_i that would obtain if each type of oscillator were the only one excited, in the following manner:

Setting $$I_{i\parallel} + I_{i\perp} = F_i,$$

$$p_i = \frac{I_{i\parallel} - I_{i\perp}}{I_{i\parallel} + I_{i\perp}} = \frac{I_{i\parallel} - I_{i\perp}}{F_i}.$$

Moreover $$I_\parallel = \sum_i I_{i\parallel} \quad \text{and} \quad I_\perp = \sum_i I_{i\perp}.$$

Therefore $$\bar{p} \equiv \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} = \frac{\sum_i F_i p_i}{\sum_i F_i}. \tag{3}$$

Addition law of the polarizations of several oscillators

The total intensity of the radiation emitted by a point of the fluorescent source can be represented by three orthogonal components. Choosing for the directions of the components the co-ordinate axes, we have in the case of non-polarized radiation,

$$I_\parallel = I_\perp = i,$$

the total intensity being proportional to $3i$. If the fluorescence excited by polarized light as described, becomes now partially polarized to the extent p_i, while the total emitted intensity remains constant, Curie's law of symmetry (Perrin, 1929) shows that the total radiation is then proportional to

$$I_\parallel + 2I_\perp = 3i.$$

This requires

$$I_\parallel = i + \Delta, \tag{4}$$

$$I_\perp = i - \tfrac{1}{2}\Delta. \tag{5}$$

From the last equations, 4 and 5, together with 1, we have for the ith oscillator species in solution:

$$\frac{\Delta_i}{2} = \frac{2i_i}{3\left(\frac{1}{p_i} - \frac{1}{3}\right)},$$

$$F_i p_i = \frac{2i_i}{\left(\frac{1}{p_i} - \frac{1}{3}\right)}.$$

Defining $f_i = \dfrac{i_i}{\sum_i i_i}$ as the contribution of the ith oscillator species to the *total* fluorescent intensity, the last equation becomes

$$F_i p_i = 2\left(\sum_i i_i\right) \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}}.$$

On the other hand, from Eqn. 3,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{\sum_i F_i}{\sum_i F_i p_i} - \frac{1}{3} = \frac{\sum_i F_i p_i \left(\frac{1}{p_i} - \frac{1}{3}\right)}{\sum_i F_i p_i}.$$

Introducing in the latter the value of $F_i p_i$ just derived,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}}}. \tag{6}$$

Thus $1/\bar{p} - \frac{1}{3}$ is the harmonic mean of the quantities $1/\bar{p}_i - \frac{1}{3}$ weighed according to the contribution to the total fluorescent intensity of the solution.

Eqns. 4 and 5 refer to excitation with polarized light. In this case the electric vector of the exciting light is an axis of symmetry and $3i = I_\parallel + 2I_\perp$. If the fluorescence is excited with natural light the direction of propagation is now an axis of symmetry (Perrin, 1929) and the total radiation of the source is proportional to $3i = 2I_\parallel + I_\perp$. Eqns. 4 and 5 become now respectively,

$$I_{i\parallel} = i_i + \tfrac{1}{2}\Delta; \quad I_{i\perp} = i_i - \Delta, \tag{7}$$

and consequently

$$\frac{1}{\bar{p}_n} + \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\frac{1}{p_{in}} + \frac{1}{3}}}, \tag{8}$$

where the subscript n refers to the same quantities as before but on excitation with natural light. Perrin's law for the excitation with natural light reads

$$\frac{1}{p_n} + \frac{1}{3} = \left(\frac{1}{p_{0n}} + \frac{1}{3}\right)\left(1 + \frac{RT}{\eta V}\tau_0\right). \tag{9}$$

Therefore in the equations to follow it will only be necessary to write $1/p_n + \frac{1}{3}$ and $1/p_{0n} + \frac{1}{3}$ instead of $1/p - \frac{1}{3}$ and $1/p_0 - \frac{1}{3}$ respectively, to pass from the case of excitation with polarized light to that of excitation with natural light.

If $1/\bar{p}-\frac{1}{3}$ is plotted against T/η in the case of a system of several components the slope, obtained by differentiating Eqn. 6, is

$$\left.\begin{aligned}\frac{\mathrm{d}}{\mathrm{d}(T/\eta)}\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)&=\sum_i \frac{\mathrm{d}\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}\frac{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}{\mathrm{d}(T/\eta)},\\ \frac{\mathrm{d}\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}&=\frac{\frac{f_i}{\left(\frac{1}{p_i}-\frac{1}{3}\right)^2}}{\left(\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\right)^2}.\end{aligned}\right\} \tag{10}$$

If the polarization of the fluorescence given by the ith oscillator species when alone in solution is described by Perrin's equation,

$$\left.\begin{aligned}\frac{1}{p_i}-\frac{1}{3}&=\left(\frac{1}{p_{0i}}-\frac{1}{3}\right)\left(1+\frac{3\tau_i}{\rho_i}\right), \quad \rho_i=\frac{3\eta V_i}{RT},\\ \frac{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}{\mathrm{d}(T/\eta)}&=\left(\frac{1}{p_{0i}}-\frac{1}{3}\right)\frac{3\tau_i}{\rho_i}\left(\frac{1}{T/\eta}\right),\end{aligned}\right\} \tag{11}$$

introducing the values of the partial differentials into Eqn. 10 and performing the additions,

$$\frac{\mathrm{d}}{\mathrm{d}(T/\eta)}\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)=\frac{\sum_i \frac{f_i}{\left(\frac{1}{p_i}-\frac{1}{3}\right)^2}\left(\frac{1}{p_{0i}}-\frac{1}{3}\right)\frac{3\tau_i}{\rho_i}}{\frac{T}{\eta}\left[\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\right]^2}. \tag{12}$$

Similarly, we may calculate

$$\frac{\mathrm{d}^2}{\mathrm{d}(T/\eta)^2}\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)=\frac{-2\left[\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\left(\frac{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}{\mathrm{d}\left(\frac{T}{\eta}\right)}\right)^2\right]\left[\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\right]+2\left[\sum_i \frac{f_i}{\left(\frac{1}{p_i}-\frac{1}{3}\right)^2}\frac{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}{\mathrm{d}\left(\frac{T}{\eta}\right)}\right]^2}{\left[\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\right]^3}.$$

Replacing $\dfrac{\mathrm{d}\left(\frac{1}{p_i}-\frac{1}{3}\right)}{\mathrm{d}(T/\eta)}$ by its value given in Eqn. 11 and performing the multiplication of the series,

$$\frac{\mathrm{d}^2\left(\frac{1}{\bar{p}_i}-\frac{1}{3}\right)}{\mathrm{d}(T/\eta)^2}=\frac{-\sum_i \frac{f_i f_j}{\left(\frac{1}{p_i}-\frac{1}{3}\right)\left(\frac{1}{p_j}-\frac{1}{3}\right)}\left(\frac{3\tau_i/\rho_i}{1+3\tau_i/\rho_i}-\frac{3\tau_j/\rho_j}{1+3\tau_j/\rho_j}\right)^2}{\left(\frac{T}{\eta}\right)^2\left(\sum_i \frac{f_i}{\frac{1}{p_i}-\frac{1}{3}}\right)^3} \quad (i\neq j). \tag{12a}$$

Therefore the first derivative is always positive and the second either zero or negative and the plotting of $1/\bar{p}$ against T/η is either a straight line or a curve concave towards the T/η axis. Eqn. 11 shows also that if the oscillators differ only in the value of the limiting polarization, p_{0i}, no curvature is to be observed. It is in fact easy to deduce that in such case the observed polarization is given by the equation

$$\frac{1}{\bar{p}}-\frac{1}{3}=\frac{1}{\left(\sum_i \frac{f_i}{\frac{1}{p_{0i}}-\frac{1}{3}}\right)}\left(1+\frac{\tau_0}{\rho}\right). \qquad (12b)$$

It is found experimentally that p_0 varies with the wavelength of the exciting light. Eqn. 12*b* shows, however, that the calculated value of the relaxation time of the rotation will be the same whether the light used for the excitation is monochromatic or not. This has been experimentally demonstrated by Perrin (1929). Eqn. 12*b* also shows that if the excitation is due to more than one wavelength a change in the observed polarization may in certain cases be due to a change in the absorption spectrum of the fluorescent solution leading to a different value of $\bar{p}_0$, and not to a change in the lifetime of the excited state or the rotational relaxation time.

Molecules of different size carrying the same fluorescent oscillator

There is in this case only one lifetime of the excited state and one limiting polarization, i.e. $p_{0i}=p_0$ and $\tau_i=\tau_0$. Eqn. 6 can be written in the form

$$\frac{1}{\bar{p}}-\frac{1}{3}=\frac{1}{\sum_i \frac{f_i}{\left(\frac{1}{p_{0i}}-\frac{1}{3}\right)\left(1+\frac{3\tau_i}{\rho_i}\right)}}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{1}{\sum_i \frac{f_i}{1+\frac{3\tau_0}{\rho_i}}}.$$

Adding to both members of the last equation the quantity

$$\left(\frac{1}{p_0}-\frac{1}{3}\right)\left\{1+3\tau_0\sum_i\frac{f_i}{\rho_i}-\sum_i f_i\left(1+\frac{3\tau_0}{\rho_i}\right)\right\}=0,$$

and rearranging the right-hand side

$$\frac{1}{\bar{p}}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left\{1+3\tau_0\sum_i\frac{f_i}{\rho_i}-\left[\sum_i f_i\left(1+\frac{3\tau_0}{\rho_i}\right)-\frac{1}{\sum_i \frac{f_i}{1+\frac{3\tau_0}{\rho_i}}}\right]\right\}. \qquad (13)$$

At values of T/η, such that all values of $3\tau_0/\rho_i$ are small compared to unity, the term between the square brackets is negligible. Therefore the initial slope in the plotting of $1/\bar{p}$ against T/η is proportional to the harmonic mean of the relaxation times of the particles weighted according to their contribution to the total emitted intensity. At values of T/η such that the term between the square brackets becomes significant, the value of $1/\bar{p}-\frac{1}{3}$ is always smaller than that corresponding to the initial slope, and the relative decrease is, according to Eqn. 13,

$$\frac{\delta\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\frac{1}{\bar{p}}-\frac{1}{3}}=\frac{\sum_i f_i\left(1+\frac{3\tau_0}{\rho_i}\right)-\frac{1}{\sum_i \frac{f_i}{1+3\tau_0/\rho_i}}}{\frac{1}{\sum_i \frac{f_i}{1+\frac{3\tau_0}{\rho_i}}}}=\left(\sum_i f_i\left(1+\frac{3\tau_0}{\rho_i}\right)\right)\left(\sum_i \frac{f_i}{1+\frac{3\tau_0}{\rho_i}}\right)-1.$$

Writing

$$1=(\sum_i f_i)(\sum_i f_i)=\sum_i f_i^2+2\sum_{\substack{i\\ i\neq j}} f_i f_j$$

and performing the multiplication of the series,

$$\frac{\delta\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\frac{1}{\bar{p}}-\frac{1}{3}}=\sum_{\substack{i\\ i\neq j}}\frac{f_i f_j 9\tau_0^2\left(\frac{1}{\rho_i}-\frac{1}{\rho_j}\right)^2}{\left(1+\frac{3\tau_0}{\rho_i}\right)\left(1+\frac{3\tau_0}{\rho_j}\right)}=\sum_{\substack{i\\ i\neq j}}\frac{f_i f_j}{\left(\frac{1+3\tau_0/\rho_j}{3\tau_0/\rho_j}\right)^2\left(\frac{1}{n-1}\right)^2+\left(\frac{1+3\tau_0/\rho_j}{3\tau_0/\rho_j}\right)\left(\frac{1}{n-1}\right)}, \qquad (14)$$

in which

$$n=\frac{\rho_j}{\rho_i},\quad n\geqslant 1.$$

In the case of only two molecular species in solution the last equation reads

$$\frac{\delta\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\frac{1}{\bar{p}}-\frac{1}{3}}=\frac{f_1 f_2}{\left(\frac{1+3\tau_0/\rho_1}{3\tau_0/\rho_1}\right)^2\left(\frac{1}{n-1}\right)^2+\left(\frac{1+3\tau_0/\rho_1}{3\tau_0/\rho_1}\right)\left(\frac{1}{n-1}\right)}. \qquad (15)$$

From Eqn. 2

$$\frac{1+3\tau_0/\rho_1}{3\tau_0/\rho_1}=\frac{\frac{1}{p_1}-\frac{1}{3}}{\frac{1}{p_1}-\frac{1}{p_0}}.$$

Substituting this in Eqn. 15 we have

$$\frac{\delta\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}=\frac{f_1f_2}{\left(\frac{\frac{1}{p_1}-\frac{1}{3}}{\frac{1}{p_1}-\frac{1}{p_0}}\right)^2\left(\frac{1}{n-1}\right)^2+\left(\frac{\frac{1}{p_1}-\frac{1}{3}}{\frac{1}{p_1}-\frac{1}{p_0}}\right)\left(\frac{1}{n-1}\right)}.$$

The presence of experimentally significant curvature requires that the lowest polarizations attained depart from the values corresponding to the initial tangent by an amount

$$\frac{\delta\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)}{\frac{1}{\bar{p}}-\frac{1}{3}}\geqslant 2{\cdot}5\epsilon,$$

where ϵ is the standard error of the observations. If we assume $\epsilon=0{\cdot}02$ and $1/p_1<10$, as in any case $1/p_0\geqslant 2$, the condition for observable curvature becomes

$$\frac{f_1f_2}{\frac{25}{16}\left(\frac{1}{n-1}\right)^2+\frac{5}{4}\left(\frac{1}{n-1}\right)}\geqslant 0{\cdot}05,$$

n is a minimum if $f_1=f_2=\frac{1}{2}$, and therefore $n>\frac{3}{2}$. Thus in a solution of two spherical molecules a deviation from the linear law will only be present under the experimental conditions described if the ratio of the relaxation times of the rotation is greater than $\frac{3}{2}$, and conversely if one molecular species carry two different oscillators in equal amounts a deviation from the linear law requires a similar ratio for the lifetimes of the excited state.

Effect of the presence of a fluorescent impurity of low molecular weight

In the case of two spherical molecules of relaxation times ρ_1 and ρ_2 Eqn. 13 becomes

$$\frac{\frac{1}{\bar{p}}-\frac{1}{3}}{\frac{1}{p_0}-\frac{1}{3}}=1+\left[\frac{3\tau_0}{\rho_2}(f_1q+f_2)\frac{\left(1+\frac{3\tau_0}{\rho_2}\left(\frac{q}{f_1q+f_2}\right)\right)}{\left(1+\frac{3\tau_0}{\rho_2}(f_1+f_2q)\right)}\right], \tag{16}$$

with $q=\rho_2/\rho_1$, $q\leqslant 1$. The slope S on the plotting of $\frac{1/\bar{p}-\frac{1}{3}}{1/p_0-\frac{1}{3}}$ against T/η can be obtained by differentiating Eqn. 16:

$$S=\frac{(f_1q+f_2)\frac{3\tau_0}{\rho_2}+2q\left(\frac{3\tau_0}{\rho_2}\right)^2+q(f_1+f_2q)\left(\frac{3\tau_0}{\rho_2}\right)^3}{\left(1+\frac{3\tau_0}{\rho_2}(f_1+f_2q)\right)^2\left(\frac{T}{\eta}\right)}. \tag{17}$$

The tangent to the curve at a given value of T/η cuts the ordinate axis at the value $1/p_0'$ defined by the equation

$$\frac{\frac{1}{p_0'}-\frac{1}{p_0}}{\frac{1}{p_0}-\frac{1}{3}}=\frac{\frac{1}{\bar{p}}-\frac{1}{3}}{\frac{1}{p_0}-\frac{1}{3}}-S\frac{T}{\eta}=\frac{\left(\frac{3\tau_0}{\rho_2}\right)^2(1-q)^2f_1f_2}{\left(1+\frac{3\tau_0}{\rho_2}(f_1+f_2q)\right)^2}. \tag{18}$$

If $q\ll 1$ and $3\tau_0/\rho_2\gg 1$ we have the case in which a macromolecule and a low molecular weight impurity coexist in solution. Eqn. 18 shows that then a very simple relation obtains approximately between the relative increase in the intercept and the contribution of the impurity to the total fluorescent emission, namely:

$$f_2\simeq\frac{\frac{1}{p_0'}-\frac{1}{p_0}}{\frac{1}{p_0'}-\frac{1}{3}}. \tag{19}$$

Moreover, if $\frac{3\tau_0}{\rho_2}f_1\gg 1$,

$$\frac{\frac{3\tau_0}{\rho_2}(f_1q+f_2)}{1+\frac{3\tau_0}{\rho_2}(f_1+f_2q)}\simeq\frac{f_2}{f_1}, \tag{20}$$

and the right-hand side of Eqn. 16 reduces to

$$\frac{1}{f_1}\left(1+\frac{3\tau_0}{\rho_1}\right).$$

From the last, and Eqn. 19, we have

$$\left(\frac{1}{\bar{p}}-\frac{1}{3}\right)\simeq\left(\frac{1}{p_0'}-\frac{1}{3}\right)\left(1+\frac{3\tau_0}{\rho_1}\right). \tag{21}$$

Eqn. 21 has the same form as 2, p_0' taking the place of p_0. Therefore the linear law is followed over the range of values of T/η such that $(3\tau_0/\rho_2)f_1\gg 1$ and the relaxation time calculated from the slope and intercept is the true relaxation time of the rotation of the macromolecule.

Polarization of the fluorescence emitted by ellipsoidal molecules carrying randomly oriented oscillators

Perrin (1936) has developed the theory of the polarization of the fluorescence emitted by molecules of any shape. There appears to be no simple theory for ellipsoids of revolution. The value of p is in that case an explicit function not only of the principal relaxation times of the rotation, but also

of the three angles determined by the oscillators of absorption and emission of light and the axis of revolution of the ellipsoid.

The general equation (Perrin, 1929),

$$\frac{1}{p}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{2}{3\overline{\overline{\cos^2\omega}}-1}, \qquad (22)$$

where $\overline{\overline{\cos^2\omega}}$ is the average cosine square of the angle swept by the emission oscillator between the times of absorption and emission of light, may, however, be applied in certain cases. If OA (Fig. 1) denotes the

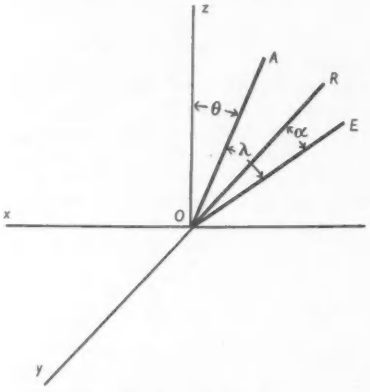

Fig. 1. Explanation in the text.

direction of the oscillator of absorption, OE the direction of the emission oscillator and OR the major axis of the ellipsoid, for the last equation to be valid it is necessary that $\overline{\overline{\cos^2\omega}}$ be independent of azimuth about OE. This requires all positions of the ellipsoid with OE in a particular direction to have equal weight. The weight of each position is given by $\cos^2\theta$, where θ is the angle between the absorption oscillator and the electric vector of the exciting light Oz. The condition that all positions of the molecule obtained by rotation about OE have equal weight can only be fulfilled in two particular instances:

(*a*) OA and OE are coincident;

(*b*) OA and OE make a fixed angle λ between them, but for a given direction of OE and OR all azimuths of OA about OE are equally probable. Clearly this is impossible if the axis of revolution of the molecule makes fixed angles with the oscillators, but it will represent the actual case when a small fluorescent molecule is attached to a much larger non-fluorescent one by adsorption or covalent bonding, provided there is no particular orientation of the molecules with respect to each other. If the larger molecule is an ellipsoid of revolution, $\overline{\overline{\cos^2\omega}}$ will be a function of the angle α between OR and OE and the two principal relaxation times of the rotation of the ellipsoid. Moreover, it will be necessary to sum over all values of α since random orientation of the second molecule with respect to the first has been assumed. By application of Eqn. 6 we then have

$$\frac{1}{p}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{2}{\frac{6}{\pi}\int_0^{\frac{1}{2}\pi}\overline{\overline{\cos^2\omega}}\,(\alpha)\,\mathrm{d}\alpha-1}, \qquad (23)$$

where $$\overline{\overline{\cos^2\omega}}\,(\alpha)=\frac{1}{\tau_0}\int_0^{\infty}\overline{\cos^2\omega}\,(\alpha,t)\,\mathrm{e}^{-t/\tau_0}\,\mathrm{d}t. \qquad (24)$$

Calculation of $\overline{\overline{\cos^2\omega}}$. Consider two positions of the ellipsoid; in the first the principal axes of the ellipsoid are coincident with the co-ordinate axes and the components of the oscillators of emission along the co-ordinates are $\cos\alpha$, $\cos\beta$ and $\cos\gamma$ respectively. After a rotation about the centre the components are $\cos\alpha'$, $\cos\beta'$ and $\cos\gamma'$. Therefore ω, the angle determined by the two directions of the oscillator of emission, is defined by,

$$\cos\omega=\cos\alpha\cos\alpha'+\cos\beta\cos\beta'+\cos\gamma\cos\gamma'.$$

If $\cos_{ij}$ ($i=1, 2, 3$; $j=1, 2, 3$) denotes the angle between the direction of a principal axis i of the ellipsoid in its second position and an axis j in the first,

$$\begin{aligned}\cos^2\omega=[&\cos^2\alpha\cos_{11}+\cos\alpha\cos\beta\cos_{21}\\&+\cos\alpha\cos\gamma\cos_{31}+\cos\alpha\cos\beta\cos_{12}\\&+\cos^2\beta\cos_{22}+\cos\beta\cos\gamma\cos_{32}\\&+\cos\alpha\cos\gamma\cos_{13}+\cos\beta\cos\gamma\cos_{23}\\&+\cos^2\gamma\cos_{33}]^2.\end{aligned}$$

Taking into account that the mean value of the double products of the form $\overline{\cos_{ij}\cos_{jk}}$, where one or more indices appear only once are null by symmetry (Perrin, 1936), and setting

$$\cos^2\alpha=z,\quad \cos^2\beta=\cos^2\gamma=\tfrac{1}{2}(1-z),$$

we have

$$\begin{aligned}\overline{\cos^2\omega}=z^2\overline{\cos^2_{11}}+&\frac{(1-z)^2}{4}(\overline{\cos^2_{22}}+\overline{\cos^2_{33}})\\&+z(1-z)(\overline{\cos^2_{21}}+\overline{\cos^2_{31}}+\overline{\cos_{11}\cos_{22}}\\&+\overline{\cos_{12}\cos_{21}}+\overline{\cos_{11}\cos_{33}}+\overline{\cos_{13}\cos_{31}}\\&+\frac{(1-z)^2}{2}(\overline{\cos^2_{32}}+\overline{\cos_{22}\cos_{33}}+\overline{\cos_{23}\cos_{32}}),\end{aligned} \qquad (25)$$

where the bar denotes average value.

To obtain $\overline{\cos^2\omega}\,(\alpha,t)$ a function of the time t, we introduce the values calculated by Perrin (1936) for the average cosine square and double products of the angles determined by the axes of the ellipsoid of

revolution submitted to brownian movement. These are

$$\left.\begin{aligned}
&\overline{\cos^2_{11}} = \tfrac{1}{3} + \tfrac{2}{3}\,e^{-3t/\rho_1},\\
&\overline{\cos^2_{22}} = \overline{\cos^2_{33}} = \tfrac{1}{3} + \tfrac{1}{6}\,e^{-3t/\rho_1} + \tfrac{1}{2}\exp\left[-\left(\frac{4}{\rho_2}-\frac{1}{\rho_1}\right)t\right],\\
&\overline{\cos^2_{12}} = \overline{\cos^2_{21}} = \overline{\cos^2_{13}} = \overline{\cos^2_{31}} = \tfrac{1}{3} - \tfrac{1}{3}\,e^{-3t/\rho_1},\\
&\overline{\cos^2_{23}} = \overline{\cos^2_{32}} = \tfrac{1}{3} + \tfrac{1}{6}\,e^{-3t/\rho_1} - \tfrac{1}{2}\exp\left[-\left(\frac{4}{\rho_2}-\frac{1}{\rho_1}\right)t\right],\\
&\overline{\cos_{22}\cos_{11}} + \overline{\cos_{12}\cos_{21}} = \overline{\cos_{33}\cos_{11}} + \overline{\cos_{13}\cos_{31}}\\
&\qquad = \exp\left[-\left(\frac{2}{\rho_1}+\frac{1}{\rho_2}\right)t\right],\\
&\overline{\cos_{22}\cos_{33}} + \overline{\cos_{23}}\;\overline{\cos_{32}} = \exp\left[-\left(\frac{4}{\rho_2}-\frac{1}{\rho_1}\right)t\right],
\end{aligned}\right\} \qquad (26)$$

ρ_1 and ρ_2 are the relaxation times of the rotation of the ellipsoid about the long and short axis respectively. By introduction of these values in Eqn. 25 and integration of Eqn. 24:

$$\overline{\overline{\cos^2}}\,\omega(\alpha) = \frac{1}{3} + \frac{(1-3z)^2}{6}\,\frac{1}{\left(1+\frac{3\tau_0}{\rho_1}\right)} + \frac{(1-z)^2}{2}\,\frac{1}{\left(1+\left(\frac{4}{\rho_2}-\frac{1}{\rho_1}\right)\tau_0\right)} + 2z(1-z)\,\frac{1}{\left(1+\left(\frac{2}{\rho_1}+\frac{1}{\rho_2}\right)\tau_0\right)}, \qquad (27)$$

and finally integrating Eqn. 23 after introducing $\overline{\overline{\cos^2}}\,\omega(\alpha)$

$$\frac{1}{p}-\frac{1}{3} = \left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{AB}{C}\left(\frac{8}{3+6\frac{B}{C}-\frac{B^2}{C^2}}\right), \qquad (28)$$

where

$$A = 1+\frac{3\tau_0}{n_1\rho_0}, \quad B = 1+\left(\frac{4}{n_2}-\frac{1}{n_1}\right)\frac{\tau_0}{\rho_0}, \quad C = 1+\left(\frac{2}{n_1}+\frac{1}{n_2}\right)\frac{\tau_0}{\rho_0},$$

$$\frac{\rho_1}{\rho_0} = n_1, \quad \frac{\rho_2}{\rho_0} = n_2, \quad \rho_0 = \frac{3\eta V_e}{RT}, \quad V_e = \text{volume of the ellipsoid.}$$

The last equation can also be written

$$\frac{1}{p}-\frac{1}{3} = \left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{\left(1+\frac{3\tau_0}{n_1\rho_0}\right)\left(1+\left[\frac{4}{n_2}-\frac{1}{n_1}\right]\frac{\tau_0}{\rho_0}\right)}{1+\frac{1}{2}\left(\frac{5}{n_2}+\frac{1}{n_1}\right)\frac{\tau_0}{\rho_0}+\frac{9}{8}\,\frac{\frac{\tau_0^2}{\rho_0^2}\left(\frac{1}{n_2}-\frac{1}{n_1}\right)^2}{\left(1+\left[\frac{2}{n_1}+\frac{1}{n_2}\right]\frac{\tau_0}{\rho_0}\right)}}, \qquad (29)$$

which clearly shows that on plotting $1/p-\frac{1}{3}$ against τ_0/ρ_0 or, which is equivalent, against T/η, the ratio of the initial slope to that of the sphere of the same volume is

$$\frac{S}{S_0} = \frac{1}{2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right), \qquad (30)$$

the harmonic mean of the ratios of the relaxation times of the ellipsoid to the relaxation time of the sphere of the same volume. If the last term of the denominator of Eqn. 29 is neglected the equation describes within 1% the depolarization due to molecules of any elongation up to $\tau_0/\rho_0 = 0{\cdot}13$ and within 5% up to $\tau_0/\rho_0 = 0{\cdot}5$. A better and more interesting approximation is given by the equation

$$\left.\begin{aligned}
&\frac{1}{p}-\frac{1}{3} = \left(\frac{1}{p_0}-\frac{1}{3}\right)\left(1+\frac{3\tau_0}{\rho_0 n_p}\left[\frac{1+\frac{3\tau_0}{\rho_0 n_a}}{1+\frac{3\tau_0}{\rho_0 n_p}}\right]\right),\\
&\frac{1}{n_p} = \frac{1}{2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right), \quad n_a = \frac{n_1+n_2}{2}.
\end{aligned}\right\} \qquad (31)$$

The last is obtained immediately from Eqn. 6 in the case of two spherical molecules present in equal amounts ($f_1 = f_2 = \frac{1}{2}$) and having relaxation times ρ_1 and ρ_2. The random distribution of the directions of the oscillators in an ellipsoidal molecule result in almost complete uncoupling of the effects of both relaxation times, so that qualitatively and almost quantitatively the situation is much the same as if both relaxation times belonged to different molecules.

Depolarization by flat and elongated molecules

If the molecule is a flat ellipsoid of revolution its two principal relaxation times never differ between themselves by more than 10% (Perrin, 1934). Consequently, on plotting $1/p$ against T/η the departure from linearity is negligible and a flat ellipsoidal molecule cannot be distinguished from a spherical molecule. On the other hand, the two relaxation times of the rotation of an elongated ellipsoid may differ greatly and therefore a curvature concave towards the T/η axis may be obtained.

Fig. 2 shows the plot of $(1/p-\frac{1}{3})/(1/p_0-\frac{1}{3})$ against τ_0/ρ_0 calculated from Eqn. 28 for ellipsoids of revolution of increasing axial ratio; n_1 and n_2 have been calculated from the equations of Perrin (1934). There is a mistake in the values of n_2 in equations 96 and 96 *bis* in Perrin's (1934) paper. $2\rho^2-1$ must be changed into $1-2\rho^2$, the mistake coming from equation 95 where the coefficient of S in the denominator of n_2 should be $b^2a(a^2-2b^2)$ instead of $b^2a(2b^2-a^2)$. From Fig. 2 it appears that the curvature is pronounced only if the accessible range of values of τ_0/ρ_0 is sufficiently large. Thus if $\tau_0/\rho_0<0{\cdot}5$ no elongation smaller than 6 is likely to be detected, and if $\tau_0/\rho_0<0{\cdot}2$ any elongation will fail to produce curvature.

times may be approximated by the use of Eqn. 16. In order to see the significance of f_1 and f_2 we may consider the likely case where the attached molecule executes a very rapid rotation restricted to a few degrees, independent of the slow rotation of the macromolecule. Since $3\tau_0/\rho_2 \gg 1$, the plot of $1/p$ against T/η will yield a straight line extrapolating to $1/p_0' > 1/p_0$. After Eqn. 22 if θ is the average amplitude of the rapid rotation of the small molecule,

$$\frac{1}{p_0'}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\frac{2}{3\cos^2\theta-1}. \qquad (32)$$

So that Eqn. 19 yields

$$f_2=\tfrac{3}{2}\sin^2\theta,$$

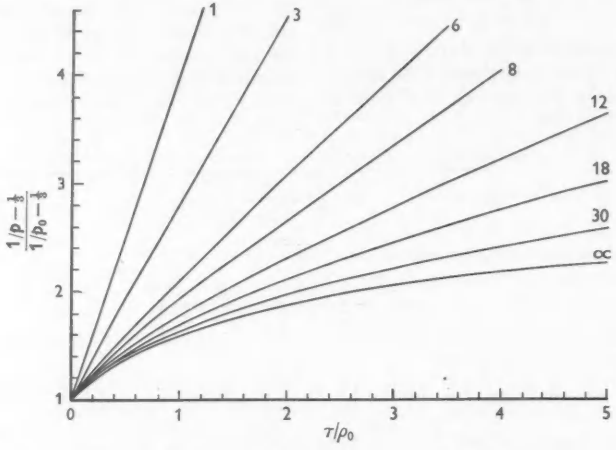

Fig. 2. Theoretical curves (Eqn. 28) for prolate ellipsoids of revolution. The number of each curve gives

$$\text{the elongation}=\frac{\text{long axis}}{\text{short axis}}.$$

It may be concluded that in the case of molecules not very asymmetrical, i.e. the globular proteins, the simple Eqn. 1 should apply if ρ_0 is substituted by ρ_p, the harmonic mean of the principal relaxation times of the rotation.

Depolarization of the fluorescence by intramolecular rotation

The considerations that lead us to the use of Eqn. 22 allow a qualitative treatment of the case in which the small molecule attached to the macromolecule has a certain freedom of rotation with respect to the latter. If the macromolecule follows the linear law, i.e. if its depolarization can be described as resulting from one relaxation time $\rho_p=\rho_1$ and the small molecule has relaxation time ρ_2 about its conjugating bond or one nearby to it, the composition of the effects of these two relaxation relating f_2 to the amplitude of the rapid rotation. If $(3\tau_0/\rho_2)f_1 \gg 1$ the linear law is followed, just as in the case of an impurity of low molecular weight, and the relaxation time calculated from the slope and intercept is the relaxation time of the rotation of the macromolecule as a whole.

It is evident from the preceding discussion that the study of the polarization of the fluorescence alone cannot distinguish between the effects of intramolecular rotations and the presence of a small amount of an impurity of low molecular weight. In both cases the net effect is a decrease in the value of p_0, so that the relaxation time of the rotation of the macromolecule can be calculated without difficulty.

In the preceding discussion we have assumed that the amplitude of the internal rotations does not change with temperature. It is conceivable, however, that the amplitude allowed to such rotations

may increase due to thermal breaking of intramolecular bonds, i.e. f_2 increases with the temperature. The last two equations then give

$$\frac{1}{p}-\frac{1}{3}=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left(\frac{1}{1-f_2(T)}\right)\left(1+\frac{3\tau_0}{\rho_v}\right).$$

On plotting $1/p$ against T/η a curvature convex towards the latter axis will appear, just as in the case of thermal dissociation of the molecule into rigid units. If a maximum value of f_2 is reached a straight line (s_2) or a curve concave towards the T/η axis may again obtain at higher temperature. If a definite slope is finally reached $1/p_0'$ can be determined by extrapolation. If $1/p_0=1/p_0'$ the curvature cannot be due to internal rotations and must result from molecular fission; s_2/s_1, the ratio of the initial to the final slope, is then the ratio of the average relaxation times of the original and resulting particles. If $1/p_0 \neq 1/p_0'$, and if intramolecular rotations alone are the cause of the curvature, from Eqn. 21

$$\frac{s_2}{s_1}=\frac{\frac{1}{p_0'}-\frac{1}{3}}{\frac{1}{p_0}-\frac{1}{3}} \qquad (33)$$

must be satisfied within the limits of the experimental error.

EXPERIMENTAL METHOD

The arrangement used for the determination of the polarization of the fluorescence is shown in Fig. 3. Light from a mercury arc is made parallel and then polarized by the

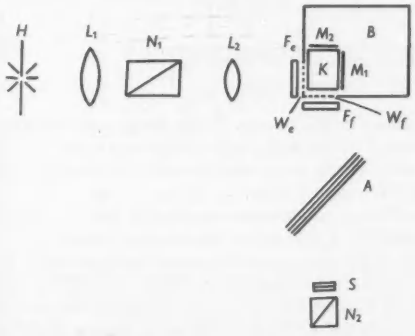

Fig. 3. Experimental arrangement for the determination of the polarization of the fluorescence. H, Hg discharge lamp; L_1 and L_2, lenses ($f_1=8$ cm.; $f_2=12{\cdot}5$ cm.); N_1 and N_2, Nicol prisms; K, cell containing the fluorescent solution; B, water bath with glass windows; F_e and F_f, complementary filters; A, Arago compensator; S, Savart polariscope; M_1 and M_2, mirrors; W_e and W_f, windows.

Nicol prism N_1. A suitable filter F_e is placed between the latter and the cell K containing the fluorescent solution. This is an ordinary glass cell with parallel faces of 1 cm. depth; it stands inside a small square water bath of some 50 ml. capacity provided with glass windows for the exciting and fluorescent light, W_e and W_f respectively. The temperature of the bath is kept constant by means of a small heater and rheostat, or by cooling with ice as the case requires. The filter for the fluorescent light F_f, the Arago compensator A of four glass plates, the Savart polariscope S and its Nicol N_2 are mounted on a small optical bench at right angles to that carrying the lenses, the Nicol N_1 and the bath. The Arago compensator stands on a graduated circle and a lateral vernier allows readings of 0·1 degree. The Savart plate is mounted so that it can be tilted slightly. In this way the interference fringes can be seen moving over the field with a good improvement in sensitivity. Two small mirrors, M_1 and M_2, can be placed against the back and side of the glass cell respectively, as described by Perrin (1929). The first increases the number of excited molecules and makes the field more homogeneous while the second increases the light reaching the eye by a factor of nearly two.

Calibration of the compensator of Arago

A source of known polarization is required. This is provided by the light reflected from the face of a glass prism of known refractive index, at different incidence angles. The polarization is given by the well-known Fresnel equations (Gaviola & Pringsheim, 1924). A check on the consistency of the calibration curve can easily be done by the following method:

Let the electric vector of the linearly polarized exciting light make an angle θ with the Oz direction instead of being coincident with it as we have assumed till now. The fluorescent light emitted in the Oy direction has components $I_{\theta\parallel}$ and $I_{\theta\perp}$ related to $I_\parallel$ and $I_\perp$, the components that obtain at $\theta=0$, in the following manner:

$$I_{\theta\parallel}=I_\parallel\cos^2\theta+I_\perp\sin^2\theta,$$

$$I_{\theta\perp}=I_\perp,$$

$$\frac{1}{p_\theta}=\frac{I_\parallel\cos^2\theta+I_\perp(1+\sin^2\theta)}{(I_\parallel-I_\perp)\cos^2\theta}=\frac{1}{p}+\tan^2\theta\,\frac{2I_\perp}{I_\parallel-I_\perp},$$

and remembering that $I_\parallel/I_\perp=\frac{1+p}{1-p}$, we have

$$\frac{1}{p_\theta}=\frac{1}{p}+\left(\frac{1}{p}-1\right)\tan^2\theta. \qquad (34)$$

If a solution showing considerable polarization of the fluorescence, e.g. fluorescein in glycerol, is excited with light vibrating at a variable angle θ to the Oz axis, and the observed values of $1/p_\theta$ are plotted against $\tan^2\theta$ a straight line is obtained, the slope and intercept of which should be in the relation indicated by Eqn. 34 if no systematic error is present in the calibration of the compensator.

Some sources of error in the determination of the polarization

From Eqn. 34 it is found that if $\theta=2°$ the polarization differs by less than 1/1000 from that of $\theta=0$. Therefore the inaccuracy in setting the plane of polarization of the exciting light should not result in any appreciable error in the determined polarization.

Convergence of the exciting beam. If a solution is illuminated with a plane polarized, parallel beam BO (Fig. 4), the direction of which has latitude θ and longitude ϕ, and the direction of the electric vector of which makes an angle θ with Oz, an observer receiving light from the source along Oy would record a polarization $p(\theta, \phi)$ defined by

$$p(\theta,\phi)=\frac{i_\parallel - i_\perp}{i_\parallel + i_\perp},$$

where $i_\parallel$ and $i_\perp$ are the emitted intensity components polarized along Oz and Ox respectively. The polarization p observed at right angles to the directions of propagation and vibration of the beam being as usual

$$p \equiv \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}.$$

From Fig. 4,

$$\left.\begin{aligned} i_\parallel &= I_\parallel \cos^2\theta + I_\perp \sin^2\theta, \\ i_\perp &= I_\parallel \cos^2\phi \sin^2\theta + I_\perp(\cos^2\theta\cos^2\phi + \sin^2\phi). \end{aligned}\right\} \quad (35)$$

The polarization $\overline{p}(\theta, \phi)$ observed on excitation by a convergent beam of homogeneous intensity, of maximum latitude θ and maximum longitude ϕ is, by Eqn. 3,

$$\overline{p}(\theta\phi)=\frac{\int_0^\phi\int_0^\theta (i_\parallel - i_\perp)\,\mathrm{d}\theta\,\mathrm{d}\phi}{\int_0^\phi\int_0^\theta (i_\parallel + i_\perp)\,\mathrm{d}\theta\,\mathrm{d}\phi}.$$

Introducing the values of $i_\parallel$ and $i_\perp$ given by Eqn. 35 into the last one and performing the integrations,

$$\overline{p}(\theta,\phi)=\frac{(I_\parallel - I_\perp)\frac{1}{4}\left(1+3\frac{\sin 2\theta}{2\theta}-\frac{\sin 2\phi}{2\phi}+\frac{\sin 2\theta}{2\theta}\frac{\sin 2\phi}{2\phi}\right)}{(I_\parallel + I_\perp)-(I_\parallel - I_\perp)\frac{1}{4}\left(1-\frac{\sin 2\theta}{2\theta}-\frac{\sin 2\phi}{2\phi}+\frac{\sin 2\theta}{2\theta}\frac{\sin 2\phi}{2\phi}\right)}. \quad (36)$$

For conical convergence $2\theta = 2\phi = x$,

$$\frac{1}{\overline{p}(x)}=\frac{1}{p}\frac{1}{\frac{1}{4}\left(1+\frac{\sin x}{x}\right)^2}-\frac{\left(1-\frac{\sin x}{x}\right)^2}{\left(1+\frac{\sin x}{x}\right)^2}.$$

The second term can be neglected since it is smaller than 1/1000 even for $x = \frac{1}{6}\pi$, and consequently,

$$\overline{p}(x)=\tfrac{1}{4}p\left(1+\frac{\sin x}{x}\right)^2 \geqslant p\frac{\sin x}{x}.$$

Proceeding in a similar way, it is possible to show that in the case of an excitation with natural light the polarization $\overline{p}_n(x)$ is related to that observed on excitation by a perfectly parallel beam p_n, by the equation,

$$p_n(x)=p_n\left\{\frac{\sin x}{x}-\frac{1}{4}\left(\frac{1-\sin^2 x}{x^2}\right)\right\} \geqslant p_n\left\{\frac{3}{2}\frac{\sin x}{x}-\frac{1}{2}\right\}. \quad (37)$$

Therefore convergences below 10° give rise to negligible errors in the observed polarization.

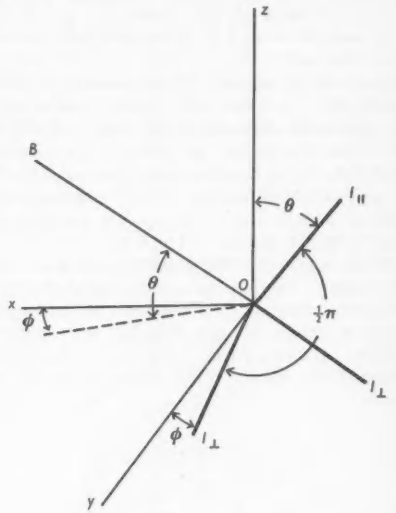

Fig. 4. Convergence of the exciting beam (for explanation see text).

Size of the source. A point in the centre of the source emits a cone of light of solid angle θ determined by the aperture of the Nicol N_2 and its distance to the source. If the Arago compensator is rotated through an angle ϕ it is easy to see that the normal to the latter makes with the divergent light angles varying from $\phi - \frac{1}{2}\theta$ to $\phi + \frac{1}{2}\theta$. It is then possible to see interference bands due to overcompensation at one end of the source before they have been fully compensated at the opposite end. It is possible to use this fact to obtain a sharp end point in the compensation by the interposition of a lens between the source and the compensator so that the necessary divergence is obtained.

Scattering of the exciting and fluorescent light. If the filters F_e and F_f for the exciting and fluorescent light are truly complementary any scattered or reflected exciting light will not reach the eye. The scattering of the fluorescent light merits further attention. Two effects have here to be considered: the lateral scattering which would result in an increase in the observed polarization and the forward scattering which should have the opposite result. As might be

expected, the first effect is negligible compared to the second, and it is found experimentally that the polarization of the fluorescence from a turbid solution is always lower than one of the same characteristics from a transparent medium. Fortunately, the Tyndall effect shown by dilute protein solutions seems to have no detectable effect on the polarization. It is a simple matter to detect in any given case whether depolarization by forward scattering takes place. With the mirror M_2 in position, and the exciting beam traversing the middle of the cell, the average path of the fluorescent light inside the solution is about twice the path in the absence of the mirror. If the polarization of the fluorescence is the same with and without the mirror any depolarization by turbidity can be excluded.

SUMMARY

1. A simple addition law for the polarizations of several fluorescent components in solution has been derived.

2. It is shown that a system of components which differ in molecular size or in their lifetime of the excited state, but which follow independently Perrin's law of depolarization give in the plot of $1/p$ against T/η a curve concave with respect to the latter axis. (p = polarization; T = absolute temperature; η = viscosity of the solvent.)

3. An extension of Perrin's theory of depolarization to the case of ellipsoidal molecules carrying randomly oriented oscillators of absorption and emission is described. In such case the polarization of the fluorescence is an explicit function of the principal relaxation times of the rotation of the ellipsoid, the lifetime of the excited state and the limiting polarization.

4. A qualitative treatment of the depolarization by intramolecular rotations is given.

5. The experimental determination of the polarization is described and some causes of error discussed in detail.

The author wishes to express his thanks to Prof. F. Perrin and Dr H. E. Daniels for their suggestions.

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Polarization of the Fluorescence of Macromolecules

2. FLUORESCENT CONJUGATES OF OVALBUMIN AND BOVINE SERUM ALBUMIN

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In the preceding paper (Weber, 1952) the theory of the polarization of the fluorescence given by macromolecules in solution carrying randomly oriented linear oscillators has been examined. If the relaxation time of the rotation of proteins is to be obtained by this method, the fluorescent oscillators must be rigidly bound to the molecule. Proteins as such are wholly non-fluorescent, but the radiation emitted by a stable complex of the protein with a small fluorescent molecule should have the same optical characteristics as a fluorescence of the protein molecules themselves.

Such a stable fluorescent complex can in theory be obtained in several ways: (i) by coupling of a small molecule through a covalent bond and subsequent elimination of the non-coupled fluorescent molecules by the ordinary methods of protein purification; (ii) by adsorption equilibrium of fluorescent molecules on the protein. It will be necessary in the latter case to determine the fraction bound to the protein by an independent method or to consider only the polarization values obtained under conditions such that the whole of the dye is bound to the protein. This last condition requires an affinity of the protein for the fluorescent molecule which is

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lacking in most proteins with the conspicuous exception of serum albumin. (iii) By use of a low molecular weight substance which becomes fluorescent on adsorption to the protein. This method is free from the objections of the preceding one, and also from the objection that the protein is being chemically modified as is the case when a fluorescent molecule is attached by covalent bond. We shall show in a later paper that a family of substances exists which, although non-fluorescent in ordinary water solution, become strongly fluorescent when adsorbed.

The present paper refers to the first possibility, namely observations on stable fluorescent conjugates.

It has already been shown (Weber, 1952) that the radiation emitted by a collection of small fluorescent units attached with random orientation to macromolecules which are flat ellipsoids or prolate ellipsoids of small elongation follows a law of depolarization analogous to that first proposed by Perrin (1926) for spherical molecules, namely

$$\frac{1}{p} \mp \frac{1}{3} \simeq \left(\frac{1}{p_0} \mp \frac{1}{3}\right)\left(1 + \frac{3\tau_0}{\rho_h}\right). \qquad (1)$$

Here p is the degree of polarization of light emitted at right angles to the direction of the excitation, τ_0 the lifetime of the excited state of the fluorescence, and ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule. The negative signs correspond to excitation with polarized light vibrating normally to the directions of excitation and observation, the positive signs to excitation by natural light; p_0 is an empirical constant dependent often on the exciting wavelength (but not on τ_0) and in the case of macromolecules (Weber, 1952) perhaps dependent on the existence of intramolecular rotations having a relaxation time much shorter than ρ_h and largely independent of the viscosity of the solvent. This paper describes the preparation and properties of conjugates obtained by reaction of 1-dimethylaminonaphthalene-5-sulphonyl chloride with ovalbumin and bovine serum albumin. Measurements of the polarization of the radiation at different temperatures allow the validity of Eqn. 1 to be tested and the values of ρ_h in the two conjugates and in the same conjugate under different conditions to be compared.

In order to obtain reliable results the following requirements must be met:

(1) The coupling should result in a minimum of chemical change of the protein molecule.

(2) As far as possible only one type of bond should be formed between the protein and the coupled molecule. The formation of comparable amounts of different bonds may result in the protein carrying oscillators with widely different lifetimes of the excited state.

(3) The conjugate must have a fluorescent efficiency comparable to that of the non-conjugated fluorescent substance. If the excess of the latter, which has failed to couple, has a much stronger fluorescence than the conjugate, a very exhaustive purification of the protein will be necessary in order to obtain reproducible results.

(4) A conjugating bond more stable than those responsible for the macromolecular structure of the protein may be required. Such a bond must be stable over the whole range of pH at temperatures below 100°, if the effect of temperature up to that capable of inducing denaturation is to be explored.

(5) It is desirable that the fluorescence should persist at high and low pH values. This results in a serious restriction, since in many substances the fluorescence disappears in acid or alkaline solution. If additional corrections are to be avoided in the comparisons of relaxation times obtained at different pH values, it is necessary that the quenching by acid or alkali should not affect the lifetime of the excited state of the fluorescence. No general rule can be given on this point. For example, the quenching of the fluorescence of riboflavin by acid is accompanied by decrease in the lifetime of the excited state, though not that of eosin (Weber, 1948).

The evidence to be presented in this paper shows that these conditions are met by the conjugates of serum and egg albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride. The coupling does not induce any observable denaturation of ovalbumin, as judged by the solubility at the isoelectric point; experiments with fumarase and ribonuclease indicate that the enzyme activity of conjugates containing 1–3 mol. of naphthalene per mol. of protein is comparable to that of the untreated protein. Although the formation of only one type of linkage cannot at present be demonstrated, this is rendered very likely by the large difference in affinity of the sulphonyl chloride for the —OH and =NH as compared to $—NH_2$ groups. The stability of the $—SO_2NH—$ bond is well known. In no experiment was there any evidence found of the breaking down of the conjugate as shown by the appearance of the free naphthalenesulphonic acid. The fluorescence of the conjugates was not conspicuously affected by changes in the pH between 1·6 and 14.

From Eqn. 1 it is easily shown that if $p_{min.}$ denotes the smallest polarization that can be measured with standard error ϵ the range of values of the ratio ρ_h/τ_0 that can be measured with that precision is given by

$$\frac{3+p_0}{3\epsilon} > \frac{\rho_h}{\tau_0} > \frac{3+p_0}{\dfrac{p_0}{p_{min.}} - 1}.$$

In the sulphonamido conjugates studied p_0 (excitation with natural light) = 0·25; $p_{min.} = 0·1$; and $\epsilon = 0·02$. Therefore,

$$55 > \frac{\rho_h}{\tau_0} > 2.$$

If τ_0 is of the order of 10^{-8} sec. we may expect measurable variations in the polarization for molecules having relaxation times of 10^{-7} sec. order of magnitude. In this range fall the harmonic means of the relaxation times of the rotation of most of the globular proteins studied by the dielectric dispersion method (Oncley, 1942).

EXPERIMENTAL

The technique and apparatus for the polarization determinations have already been described (Weber, 1952). In the experiments described here only excitation with natural light was employed. The measurements of fluorescent intensity were made with a modified Pulfrich photometer. For both the polarizations and intensity observations the exciting light (Hg arc) was filtered through a 5850 Corning glass filter, while the fluorescence was observed through a 335 Corning glass filter. The excitation was due to the Hg lines at 366, 404 and 436 mμ. The values of p_0 obtained are therefore composite values resulting from polychromatic excitation. For reasons indicated below, the viscosities of the solutions were assumed to be those of the pure solvent. The values of Bingham and White (water) and Bingham and Jackson (sucrose solutions), as given by Bingham (1922) were used in the calculations. The absorption spectra were measured with a Beckman quartz spectrophotometer.

Materials

Ovalbumin. Preparations *A* and *C* were three and five times recrystallized ovalbumin prepared by the author (*A*) and by Dr K. Bailey (*C*). Preparation *B*, obtained from Dr A. C. Chibnall, was originally prepared by Prof. R. K. Cannan. It had been stored at room temperature as dry crystals for more than 10 years. About 10% of it was insoluble at pH 4·75, 0·1 ionic strength. Only the fraction soluble at the isoelectric point was used in the preparation of the conjugate.

Serum albumin. Crystalline bovine serum albumin (Armour Laboratories Batch nos. 10,522 and 14,656) was used throughout.

Polylysine. This was prepared by Dr C. S. Hannan (in the Press) by a modification of the method of Katchalski, Grosfeld & Frankel (1948).

Sucrose was a commercial product, the reducing power of which was equivalent to 7 parts of glucose in 100,000 (Benedict). The specific rotation was $[\alpha]_D^{20°} = 66·7°$ in water (24% w/v).

1-*Dimethylaminonaphthalene-5-sulphonyl chloride* (*V*). 1-Dimethylaminonaphthalene-5-sulphonic acid (I) is easily prepared by methylation of the technical 1-aminonaphthalene-5-sulphonic acid (Fussgänger, 1902). The yield is about 80% of recrystallized acid. For the preparation of the chloride 2·5 g. of the sulphonic acid are ground in a mortar with 3·5 g. of PCl_5 and the resulting yellowish melt poured on water. The insoluble chloride is exhaustively washed with water, filtered and dried over $CaCl_2$. The crude dry powder is extracted successively with acetone and with M-Na_2CO_3. The alkaline extract contains usually 30–50% of unconverted acid. The acetone extract is diluted with 6 vol. of water whereupon the chloride separates as yellow or orange crystals. Yield: 25–40%, m.p. 69°. (Found: S, 12·0; Cl, 13·6. $C_{12}H_{12}O_2NSCl$ requires S, 11·9; Cl, 13·4%.)

The sulphonyl chloride is little affected by water and can be kept for months over $CaCl_2$ without apparent change. It is soluble in acetone, pyridine, benzene and dioxan, insoluble in water. It reacts readily with ammonia and aliphatic amines, much less readily with aniline and very slowly with water or ethanol.

1-*Dimethylaminonaphthalene-5-sulphonamide* (*II*). 1-Dimethylaminonaphthalene-5-sulphonyl chloride (240 mg.) was dissolved in 2 ml. acetone and 1 ml. of strong ammonia was added. The sulphonamide began to crystallize immediately. Yield: 200 mg. The sulphonamide was recrystallized from ethanol in long colourless needles which showed no loss of weight after 24 hr. at 115°: m.p. 215° (decomp.). (Found: C, 57·5; H, 5·1; N, 11·2; S, 12·7. $C_{12}H_{14}O_2N_2S$ requires C, 57·5; H, 5·5; N, 11·3; S, 12·8%.)

1-*Dimethylaminonaphthalene*-5-(*N-phenyl*)-*sulphonamide* (*III*). Freshly distilled aniline (0·1 ml.) and 269 mg. of sulphonyl chloride dissolved in 1·5 ml. of pyridine were heated in the water bath until no further change in colour was noticed (1–2 hr.). On addition of water a precipitate separated. This was dissolved by warming in 70% ethanol-water and, on cooling, the anilide crystallized in thin green needles. The crystals showed no loss of weight after 24 hr. at 115°, m.p. 141–142°. (Found: C, 66·3; H, 5·8; N, 8·7; S, 9·8. $C_{18}H_{18}O_2N_2S$ requires C, 66·5; H, 5·6; N, 8·6; S, 9·8%.)

1-*Dimethylaminonaphthalene*-5-(*N-benzyl*)-*sulphonamide* (*IV*). Sulphonyl chloride (220 mg.) was dissolved in 2 ml. acetone and 0·5 ml. benzylamine was added. A white mass separated immediately. The mixture was taken to dryness and the residue dissolved in a little ethanol. On addition of water the sulphonamide crystallized. The pale-green needles were washed with water and recrystallized from 50% ethanol-water. Yield 190 mg., m.p. 139°. (Found: C, 66·5; H, 5·6; N, 8·3; S, 9·5. $C_{19}H_{20}O_2N_2S$ requires C, 67·0; H, 5·9; N, 8·2; S, 9·4%.)

Preparation of the conjugates

A weight of sulphonyl chloride equal to 1–2% of the protein was dissolved in 0·5 ml. acetone and added with stirring to 10 ml. of protein solution in 0·1 M-phosphate buffer, pH 7·5, or 1% $NaHCO_3$, kept at 0–3°. The reaction mixtures were left at 0–3° until the original turbid suspension cleared (5–12 hr.). The solutions were then centrifuged to separate suspended chloride, and dialysed with stirring in the cold against 0·2 M-KCl, or 0·15 M-K_2SO_4, with frequent changes of the latter until the outer liquid showed no appreciable fluorescence. (Excitation with Hg arc through Wood's filter.) This stage was reached with most proteins after about 2 days of dialysis. Serum albumin requires a considerably longer dialysis due to the adsorption of free dye. However, the adsorbed sulphonate could be easily separated by precipitation of the protein with ethanol. The conjugate was first dialysed against 0·1 M-acetate buffer pH 4·7, and an equal volume of 80% ethanol was added slowly at 0°. The precipitate was collected and washed repeatedly with 50% ethanol-acetate buffer at 0° until a sample of the supernatant showed very weak, polarized fluorescence (due to a trace of the conjugate in

solution). Finally, the precipitate was centrifuged and redissolved in cold 0·1 M-phosphate buffer pH 7·5, and dialysed against 0·2 M-KCl. One ethanol precipitation was usually enough to liberate all the adsorbed material though occasionally a second precipitation proved necessary. The combination of the sulphonyl chloride with the protein is a heterogeneous reaction, the yield of which largely depends upon the state of division of the sulphonyl chloride. If this is sufficiently fine a comparatively stable yellow suspension is obtained which, on reacting with the protein, becomes in the course of a few hours almost or completely transparent and much paler. If the initial suspension in 5% acetone water has a chloride content greater than about 0·2 mg./ml. some of it separates as crystals and the yield decreases. If the chloride content is kept below this limit and crystallization is avoided some 50–60% of it combines with the protein as judged by spectrophotometric measurements.

RESULTS

Absorption spectrum and fluorescence of 1-*dimethylaminonaphthalene-5-sulphonic acid and derivatives*

1-Dimethylaminonaphthalene-5-sulphonic acid and its sulphonamido derivatives show an absorption band in the 300–400 mμ. region. The maximum of this band is displaced towards the ultraviolet with increase in the ionic character of the —SO_2— group as shown in Table 1, but the molar extinction coefficient of the maximum varies very little in the different derivatives. The integrated area under the above absorption band was found to be constant within 5% in substances II–IV. On this basis it must be expected that τ_0 is essentially the same in all these cases (Lewis & Kasha, 1945). The absolute values of the lifetime of the fluorescence obtained from the equation given by these authors were $1{\cdot}1$–$1{\cdot}2 \times 10^{-8}$ sec., although for the reasons pointed out by Lewis & Kasha (1945) no more than an indication of magnitude should be expected from this figure.

Table 1. *Absorption spectra and limiting polarization of* 1-*dimethylaminonaphthalene-5-sulphonic acid and derivatives*

($p_{max.}$ is the polarization observed in glycerol solution at 3°. p_0 is the limiting polarization obtained by extrapolation from measurements in glycerol at several temperatures. The concentration of the solutions in glycerol was 3×10^{-3} g./l. The absorption spectra refer to solutions in water (I), 60% ethanol (II–IV) and absolute ethanol (V).)

Substance	Position of maximum (mμ.)	Absorption coefficient (cm.²/mol.) ($\times 10^6$)	p_0	$p_{max.}$
I	312	4·55	0·250	0·245
II	329	4·05	0·267	0·255
III	340	4·40	0·267	0·260
IV	332	4·46	0·268	0·255
V	369	3·73	—	—

The fluorescence of the sulphonic acid and the substituted sulphonamides is quenched by acid, the region of rapid decrease of the fluorescence with pH being from 4 to 3. According to the theory of the quenching by collisions of the second kind (Wawilov, 1929) the quenching of a fluorescence with $\tau_0 \simeq 10^{-8}$ requires 0·1–0·01 M concentration of quencher. As this is about 50–100 times the hydrogen-ion concentration required, it may be concluded that the quenching is non-collisional. This is confirmed by measurements of the polarization of the fluorescence of acid-quenched solutions. The substances were dissolved in 1:2-dihydroxypropane, and dry hydrogen chloride gas was passed until a convenient degree of quenching was reached. The intensities before and after quenching (I_0 and I respectively) and the corresponding polarizations (p_i and p_q respectively) were recorded. The ratio of the lifetimes of the excited state before and after quenching τ_0/τ is (Sveshnikoff, 1936)

$$\frac{\tau_0}{\tau} = \frac{1/p_i - 1/p_0}{1/p_q - 1/p_0}.$$

Table 2 gives the values of I_0/I, p_i, p_q and τ_0/τ for substances I–IV. The small change in the polarization produced by the quenching reflects the long lifetime of the non-fluorescent form, the ratio Σ/τ_0 (Weber, 1948, 1950) being 50 to 100 in the different cases quoted in the table. This long-lived non-fluorescent form may be identified with the comparatively stable R-$N^+(CH_3)_2H$, the pK of which is in the neighbourhood of 4. The values of p_0, quoted in Table 1, were obtained by observation of the substances in glycerol at different temperatures.

Table 2. *Quenching of the fluorescence of* 1-*dimethylaminonaphthalene-5-sulphonic acid and derivatives*

(The concentration of the solutions was 6×10^{-3} g./l.)

	p_i	p_q	I_0/I	τ_0/τ
I	0·043	0·109	33	3·6
II	0·048	0·052	35	1·05
III	0·062	0·081	52	1·4
IV	0·053	0·070	60	1·4

The protein conjugates showed an absorption band in the 300–400 mμ. region separated from the absorption band of the protein by a well-defined minimum. Calculation of the number of molecules of sulphonamido derivative per molecule of protein has been based on the assumption that the molar absorption coefficient at the maximum of the conjugates is the same as in the sulphonamides described. An average of $4{\cdot}3 \times 10^6$ cm.²/g.mol. has been used.

A. Ovalbumin conjugates

The absorption spectrum of the conjugates is shown in Fig. 1. The maximum of the band due to the presence of the dimethylaminonaphthalene groups lies at 344 mμ. This is noticeably displaced towards the red compared with the bands of the serum albumin (maximum at 332 mμ.), and of the polylysine conjugate (maximum at 329 mμ.). This causes the solutions of ovalbumin conjugates to appear yellow while the serum albumin conjugates of similar concentration are colourless. The contents of naphthalenic groups calculated for the three conjugates studied were: $A = 1{\cdot}7$ mol. naphthalene/mol. protein; $B = 2{\cdot}0$; $C = 2{\cdot}4$. These resulted from reaction of about half the sulphonyl chloride present.

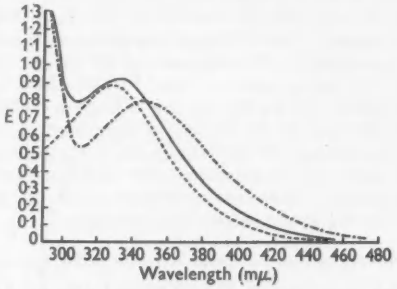

Fig. 1. Absorption spectra of conjugates. ——, Bovine serum albumin (protein concentration 0·65 %); –·–·–, ovalbumin (conjugate *B*) (protein concentration 0·48 %); ------, polylysine (concentration 0·5 %).

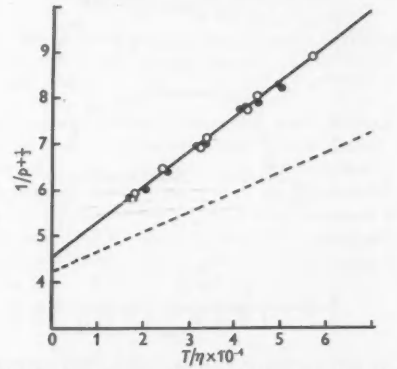

Fig. 2. Effect of temperature on the polarization of the fluorescence of ovalbumin conjugates. –○–○–, Conjugate *B*; –●–●–, conjugate *A*. The broken line is the slope observed with bovine serum albumin conjugates.

Polarization of the fluorescence

Effect of concentration. In theory, changes in the concentration of the emitting units cannot be expected to have any effect on the polarization. This was repeatedly confirmed by experiment. Solutions of ovalbumin conjugates in 0·05 M-phosphate buffer pH 7 showed no detectable change in the range of concentrations investigated, 1–0·05 % protein. Solutions of ovalbumin (0·6–1 %) dialysed against distilled water ultimately reached a pH of 4·85 and the recorded polarization was the same as with solutions in buffer, nor was this polarization changed by addition of 0·2 M-potassium chloride or by dilution down to 0·1 %.

Effect of temperature. The data are shown in Table 3 and in Fig. 2. They allow the conclusion that Perrin's law of depolarization is closely followed over the range of temperatures studied, namely 3–45°. In all the conjugates studied the plotting of $1/p$ against T/η yields straight lines with regression

Table 3. *Polarization of the fluorescence of ovalbumin conjugates at different temperatures*

Conjugate *B*. Solvent: 0·04 M-phosphate buffer, pH 6·85. Protein concentration: 0·24 g./100 ml.

Temp. (°)	T/η	p Observed	p Calculated
4·0	176	0·182	0·180
14·8	243	0·163	0·165
25·9	341	0·148	0·147
37·5	451	0·131	0·131
35·3	429	0·135	0·134
48·5	571	0·117	0·117
24·4	328	0·149	0·151
4·5	180	0·178	0·180
Solvent: 0·1 M-NaOH. Same protein concentration			
3·0	170	0·181	0·182
12·1	231	0·171	0·168
26·5	346	0·146	0·147
Conjugate *C*. Solvent: 0·04 M-phosphate buffer, pH 6·85. Same protein concentration			
3·5	171	0·182	0·181
14·9	251	0·164	0·164
8·7	206	0·175	0·174
35·0	426	0·135	0·135
25·4	337	0·150	0·148
42·4	503	0·127	0·125
42·0	498	0·127	0·126
33·9	416	0·135	0·136
5·2	184	0·181	0·179
10·0	216	0·172	0·172
18·3	179	0·163	0·158
23·9	279	0·151	0·151
31·8	395	0·139	0·139
37·8	454	0·133	0·131

The polarizations have been calculated from the equation

$$\frac{1}{p} = 4{\cdot}22 + 0{\cdot}75 \times 10^{-4}\frac{T}{\eta}.$$

The measurements are given in the order in which they were performed.

Conjugate	$1/p_0$	$b \times 10^6$	$\beta \times 10^6$
A	4·19±0·06	76·7±1·8	18·2±0·5
B	4·20±0·06	75·6±1·8	17·9±0·5
C	4·24±0·05	75 ±1·3	17·7±0·4

coefficients with a standard error of about 2%. According to Eqn. 1 the regression coefficient b contains the factor $1/p \mp \frac{1}{3}$, so that for two conjugates having intercepts $1/p_{01}$ and $1/p_{02}$

$$\frac{\rho_2}{\rho_1}=\frac{b_1}{b_2}\frac{|\,1/p_{01}\pm\frac{1}{3}}{|\,1/p_{02}\pm\frac{1}{3}}.$$

Therefore, if the linear law of depolarization is followed, the characteristic quantity for the conjugate is

$$\beta=\frac{b}{1/p_0\pm\frac{1}{3}}.$$

This is given in Table 3 for the egg albumin conjugates together with the propagated error

$$\frac{\Delta\beta}{\beta}=\sqrt{\left\{\left(\frac{\Delta b}{b}\right)^2+\left(\frac{\Delta(1/p_0+\frac{1}{3})}{1/p_0+\frac{1}{3}}\right)^2\right\}}.$$

The thermal effects were perfectly reversible. No detectable changes in the polarization were found after keeping the protein for 1 hr. at 45°.

The use of distilled water or dilute salt solutions from 2 to 50° allows a range of values of T/η between $1{\cdot}6$ and 6×10^4. To reach lower values of T/η, it is necessary to increase the viscosity of the solution by addition of a foreign substance. It is very doubtful whether the microscopic viscosity, which alone determines the resistance to the molecular rotations, can be equated in all cases with the viscosity measured by flow. To test this point the effect of electrolytes and of sucrose on the polarization was tried. Using sodium chloride and potassium chloride it was found that the increase in polarization produced by addition of electrolyte was much smaller than predicted by Eqn. 1. Concentrations below 1 M yielded polarization values which were indistinguishable from those in distilled water. Therefore in the calculation of T/η of solutions in dilute buffer ($M\leqslant0{\cdot}2$) the viscosity of the pure solvent was used in every case. Better agreement with Eqn. 1 was observed when sucrose was used to increase the viscosity of the solvent particularly in the case of neutral solutions of serum albumin as described later in this paper. The viscosity of a 60% (w/v) sucrose solution at 3–5° is of the order of poises (Bingham, 1922). From Eqn. 1, if $3\tau_0/\rho_h$ in water is 1 or less, its value in 60% sucrose should be negligible compared to 1 and the polarization observed under this condition should not differ from p_0, as determined by extrapolation, if the linear law is followed throughout the range of T/η. Native ovalbumin conjugates dissolved in 60% sucrose (w/v) yielded $p=0{\cdot}236\pm0{\cdot}004$, which is within the errors of the experiments the same as the extrapolated p_0. Therefore there is no detectable curvature and according to Eqn. I, 29,* the relaxation time calculated from the slope and intercept is ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoid. Consequently

$$\frac{\tau_0}{\rho_h}=\frac{1}{3}\frac{1/p-1/p_0}{1/p_0+\frac{1}{3}}=\frac{bT/3\eta}{1/p_0+\frac{1}{3}}=\frac{\beta T}{3\eta}$$

from Eqn. 1. If $\beta=1{\cdot}68\times10^{-5}$ and T/η at

$$25°=3{\cdot}33\times10^4,$$

then $(\tau_0/\rho_h)_{25°}=0{\cdot}186$. According to Oncley (1942), the harmonic mean of the principal relaxation times of the rotation of ovalbumin at 25° in water is $7{\cdot}55\ 10^{-8}$ sec. so that $\tau_0=1{\cdot}4\times10^{-8}$ sec. It will be convenient to assume in the future a value of $1{\cdot}4\times10^{-8}$ sec. for the lifetime of the excited state of the fluorescence of the conjugates. By introducing it in Eqn. 1 the relaxation times of the rotation of the conjugate molecules can be calculated, provided the depolarization follows the linear law.

Effect of pH *on the relaxation time of the rotation*

Changes of the pH of the solution between 1·5 and 13 produce no immediate changes in the polarization of the fluorescence. This is shown in Fig. 3

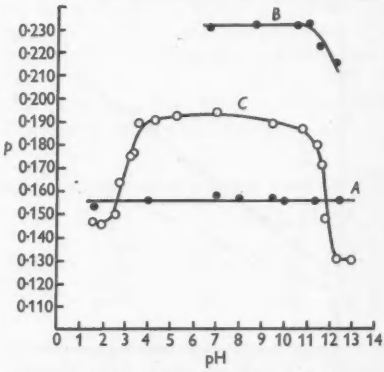

Fig. 3. Effect of pH on the polarization of the fluorescence. *A*, native ovalbumin; *B*, heat denatured ovalbumin; *C*, bovine serum albumin.

which refers to measurements done at 19°. The protein was dialysed against 0·02 M-potassium chloride and the pH adjusted, by the addition of hydrochloric acid or sodium hydroxide, to the required value which was measured by glass electrode. The polarization at each value was recorded as soon as possible afterwards, and the measurement repeated at intervals during at least 1 hr. During this time no change whatsoever was found in solutions between pH 3 and 13. At pH values between 1 and 3 a slow increase in the polarization was noticed. This increase was much faster if the solutions were kept at 37°. When the solutions kept for some time at pH 2 were adjusted to the isoelectric point a precipitate of

* This refers to Eqn. 29 of the first paper of this series (Weber, 1952). This notation is used throughout.

denatured protein appeared. The supernatant solution showed the polarization value corresponding to the native protein. This acid denaturation was studied more fully and the results are described below.

Time course of the acid denaturation

Fig. 4 shows the changes in the polarization during the course of the denaturation of 0·6% solutions of ovalbumin kept at pH 1·53–1·65. The protein was dialysed against distilled water, and then diluted to the required concentration. The

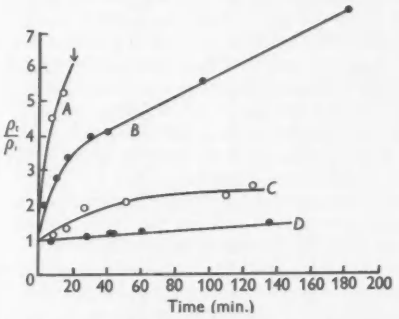

Fig. 4. Time course of the acid denaturation of ovalbumin. *A*, 0·2M-KCl, 30°; *B*, salt-free, 30°; *C*, 0·2M-KCl, 3°; *D*, salt-free, 3°.

polarization of the fluorescence was then determined at two temperatures, and finally the pH was adjusted to the required value by addition of M-hydrochloric acid. The cell was maintained at constant temperature (±0·5°) and readings were made at intervals during 2 hr. The ratio of the apparent relaxation time ρ_t to the initial relaxation time ρ_i is given by the simple equation

$$\frac{\rho_t}{\rho_i} = \frac{1/p_i - 1/p_0}{1/p_t - 1/p_0},$$

where p_i is the initial polarization and p_t its value at time t. It is assumed that the lifetime of the excited state does not change with pH. This is substantiated by the fact that in the protein solution kept at 3° in the absence of salt the value of p during the first 10 min. was the same as before the addition of the acid. In the other cases the course of the reaction shows that the value of p at zero time cannot differ greatly from that at neutral pH. Moreover, as shown by Table 2, the quenching of the fluorescence of several derivatives of 1-dimethylaminonaphthalene-5-sulphonamide by H ions does not result in any rapid change of the lifetime of the excited state of the fluorescence.

The curves of Fig. 4 show that the increase in the apparent relaxation time of the particles is much faster at the higher temperature, and faster in the presence than in the absence of salt. This applies also to the final values reached after several hours. At 30°, in the presence of salt (0·2M-potassium chloride), a copious precipitate occurred after a few minutes (arrow in Fig. 4) and no further measurements were possible.

Alkaline denaturation

The values quoted in Table 3 show that ovalbumin dissolved in 0·1M-sodium hydroxide had the same relaxation time of the rotation as at neutral pH. On adjusting the pH to the isoelectric point, the bulk of the protein separated. Therefore in alkaline solution, although the protein was denatured just as readily as in acid, no increase in the relaxation time was observed. When the isoelectric precipitate was redissolved in 0·1M-phosphate buffer, pH 7·8, considerable increase in the relaxation time of the rotation was observed. Even in 0·1M-sodium hydroxide the average relaxation time of the dissolved isoelectric precipitate was considerably higher than the native protein.

Solutions of ovalbumin kept at pH 12–13 for 24 hr. showed a decrease in the relaxation time, probably due to partial hydrolysis of the protein. Such decrease was never observed in experiments of short duration.

Urea denaturation of ovalbumin

To a solution of ovalbumin in distilled water (pH 4·9) was added 0·49 g. urea/ml. of original solution. After standing for 4 days at 0° the urea was dialysed away and the solution finally equilibrated against 0·05M-phosphate buffer, pH 7·3. The crystal clear solution had a concentration of 0·12% protein. Fig. 5 shows that the average relaxation time of the rotation of the particles had increased by about eight times as compared with that of the native protein. On heating the solution the polarization decreased as expected from the linear law.

Heat denaturation

Dilute solutions of ovalbumin (0·1–0·2%) in 0·05M-phosphate buffer, pH 7, boiled for a short time showed strong increase in the polarization of the fluorescence (Fig. 5). After boiling the solutions for 5 min. the absorption spectrum showed a maximum at 332 mμ. and was now practically coincident with the spectra of the serum albumin conjugates. If the solution was boiled for 1 min. a hybrid curve showing two maxima at 332 and 342 mμ. was obtained (Fig. 6).

The difference of absorption curves of native egg and serum albumins is not likely to be due to any difference of the covalent attachment of the sulphonamido groups. Since denatured ovalbumin, in common with other denatured proteins (Oster & Grimsson, 1949), resembles serum albumin (as

native egg albumin does not) in having a great capacity for adsorption of dyes, the identity of the absorption curve of their conjugates may be accounted for by secondary binding of the sulphonamido groups, which would be absent in conjugates

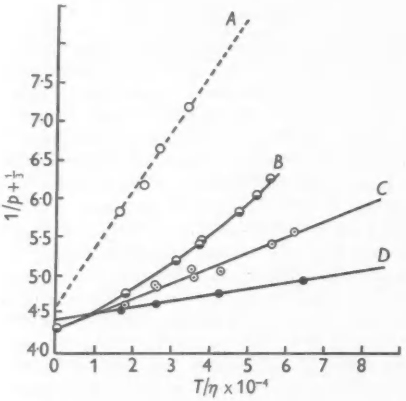

Fig. 5. Polarization of conjugates of denatured ovalbumin. *A*, ovalbumin denatured in alkali (dissolved in 0·1 M-NaOH); *B*, heat-denatured in 0·04 M-phosphate buffer, pH 7·2; *C*, denatured by urea (solvent: 0·04 M-phosphate buffer, pH 7·2); *D*, denatured by acid, salt-free, pH 1·7. The broken line is the slope corresponding to the native ovalbumin.

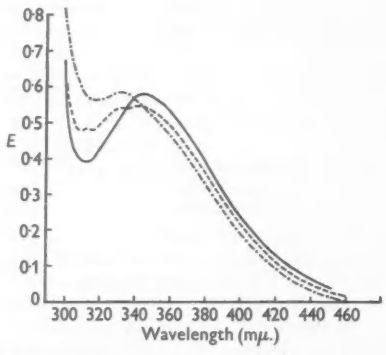

Fig. 6. Change of absorption spectrum of ovalbumin conjugates on heat denaturation. ——, native ovalbumin; - - - - - -, boiled for 1 min.; –·–·–·–, boiled for 5 min. Protein concentration 0·36 %.

of native ovalbumin. This is supported by the lower value of p_0 for native ovalbumin (0·236) than that for serum albumin (0·257), and the increase of the former value to $0·254 \pm 0·04$ on heat denaturation (measured in 60 % sucrose).

B. Bovine serum albumin conjugates

Absorption spectrum. The conjugates studied contained 1–3 mol. of naphthalene per mol. of protein of molecular weight 70 000. The absorption spectrum is shown in Fig. 1. The maximum of absorption is at 332 mμ., very nearly that obtained with a polylysine conjugate, though shifted slightly to longer wavelength. Such change has been found in the absorption spectra of many dyes adsorbed on serum albumin (Laurence, 1952). This is consistent with the secondary binding of covalently attached molecules suggested above. In the conjugate, the absorption spectrum of which is shown, there were 2·3 mol. of naphthalene per mol. of protein, resulting from the reaction of 60 % of the added chloride.

Effect of concentration on the polarization of the fluorescence. Solutions of 1–0·02 % showed no appreciable difference as regards the polarization of the fluorescence, both in distilled water (pH 5·25) and in 0·05 M-phosphate buffer, pH 7.

Temperature effect on the polarization. Solutions of conjugate at pH 6–8 followed the linear law between 3 and 50°. Above 50° a marked departure from linearity took place. The temperature effects were perfectly reversible. Keeping the protein solutions at this pH for 1 hr. at 59° did not result in any significant change in the observed polarization. Several conjugates prepared over a period of more than a year yielded entirely reproducible results. The regression coefficients of $1/p$ upon T/η varied from $4·18 \times 10^{-5}$ to $4·36 \times 10^{-5}$ in the different cases with an average of $4·25 \pm 0·1$. The value of $1/p_0$ was $3·90 \pm 0·06$. The data are given in Table 4 and in Fig. 7.

Polarization of the fluorescence in sucrose solutions. The data are shown in Table 4. It appears that the values of the polarization in 20 % (w/v) sucrose is what would be expected from Eqn. 1 if the viscosity measured by flow is introduced. The polarization calculated using $b = 4·25 \times 10^{-5}$, obtained from measurements in water, agree with the observed values within the experimental error.

Fig. 8 gives the plot of $\dfrac{1/p+\frac{1}{3}}{1/p_0+\frac{1}{3}}$ against τ_0/ρ_0; where ρ_0 is the relaxation time of a sphere of volume equal to that of the serum albumin molecule. If this has anhydrous molecular weight M and partial specific volume δ, and the hydration is H g. of water per g. of anhydrous protein

$$\rho_0 = \frac{3\eta}{RT} M (H + \delta).$$

Introducing $\tau_0 = 1·4 \times 10^{-8}$ sec. from the measurements with ovalbumin $M = 7 \times 10^4$; $\delta = 0·75$; and $H = 0·15$ from the recent measurements of Haggis, Buchanan & Hasted (1951), we have

$$\frac{\tau_0}{\rho_0} = 6·15 \times 10^{-6} \times \frac{T}{\eta}.$$

Table 4. *Polarization of the fluorescence of bovine serum albumin conjugate at different temperatures*

(Conjugate containing 2·3 mol. naphthalene/mol. protein. Solvent: 0·05 M-phosphate buffer, pH 6·77. Protein concentration: 0·12%.)

Temp. (°)	$T/\eta \times 10^{-2}$	p Observed	p Calculated
3·4	173	0·215	0·215
10·7	221	0·210	0·206
23·5	320	0·188	0·189
30·3	380	0·182	0·180
35·4	431	0·176	0·174
44·5	526	0·163	0·162
38·9	465	0·173	0·169
51·5	606	0·155	0·153
55·7	657	0·148	0·148
58·4	690	0·140	0·146
14·5	248	0·200	0·201
3·0	170	0·215	0·216
20·2	293	0·192	0·193
50·2	592	0·152	0·155
54·8	646	0·145	0·149
58·8	696	0·138	0·144
Same conjugate. Sucrose added to 20% (w/v).			
3·5	83	0·237	0·236
16·7	135	0·225	0·224
27·6	188	0·214	0·213
35·1	235	0·206	0·204
39·5	260	0·200	0·198
48·1	319	0·190	0·190
53·5	365	0·181	0·182

The polarizations have been calculated from the equation

$$\frac{1}{p} = 3{\cdot}90 + 0{\cdot}433 \times 10^{-4}\,\frac{T}{\eta}.$$

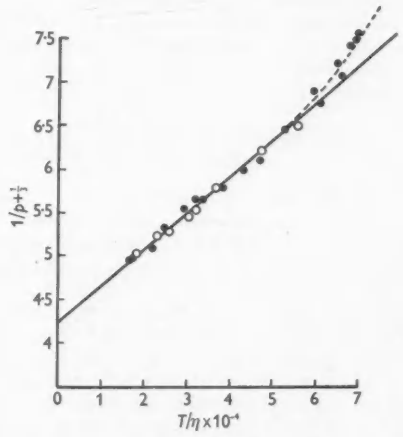

Fig. 7. The effect of temperature on the polarization of the fluorescence of bovine serum albumin conjugates. The open and filled circles correspond to two different preparations. The solid line is the regression line obtained from the observations at temperatures below 50°.

The figure shows that an axial ratio of 4 would account well for the observed values. If $H = 0{\cdot}3$ the axial ratio would be about 3 and 5 if $H = 0$.

The polarization recorded in 60% sucrose at 2° was $0{\cdot}257 \pm 0{\cdot}002$. Within the limits of the experimental error this is the same as the value obtained by extrapolation (0·256).

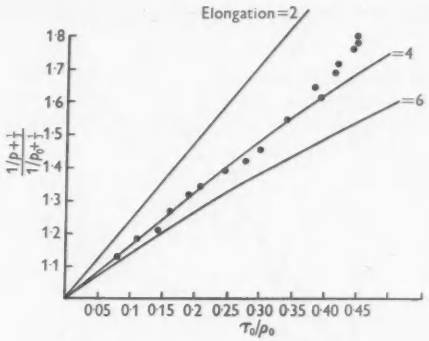

Fig. 8. Abscissa: τ_0/ρ_0. Ordinate: $\dfrac{1/p+\frac{1}{3}}{1/p_0+\frac{1}{3}}$, as explained in the text. The curves are the theoretical for ellipsoids of elongation 2, 4 and 6 (Weber, 1952).

The harmonic mean of the relaxation times of the rotation at 25° in water can be determined from Eqn. 1 in which $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec. We thus obtain $\rho_h = 1{\cdot}27 \times 10^{-7}$ sec., while Oncley (1942) gives $\rho_h = 1{\cdot}24 \times 10^{-7}$ sec. from measurements of the dielectric dispersion with horse serum albumin.

Observations on bovine serum albumin conjugate in acid solution

If a 0·1% solution of conjugate is thoroughly dialysed against distilled water and the pH adjusted to 1·8–2 by addition of a small amount of M-hydrochloric acid the polarization of the fluorescence drops from 0·196 (at 18°) to 0·147. Measurements at different temperatures at this pH yielded

$$b = (9{\cdot}68 \pm 0{\cdot}2) \times 10^{-5};$$
$$1/p_0 = 4{\cdot}08 \pm 0{\cdot}07;$$
$$\beta = (2{\cdot}2 \pm 0{\cdot}06) \times 10^{-5}.$$

In 60% sucrose $p = 0{\cdot}245$ in good agreement with the extrapolated p_0. The results are shown in Fig. 9. After neutralization by dialysis against 0·05 M-phosphate buffer, pH 7, the observed polarizations yielded $b = 4{\cdot}3 \times 10^{-5}$; $1/p_0 = 3{\cdot}95$; $\beta_0 = 1{\cdot}02 \times 10^{-5}$, showing that the changes in polarization with pH were perfectly reversible.

The intensity of the fluorescence of the salt-free conjugates at pH 2 was 60% of the intensity at pH 7, while the absorption spectrum showed no conspicuous change. The observations on the ovalbumin

conjugates, together with those on the quenching of the fluorescence of the sulphonamido derivatives, show that no change in the lifetime of the excited state is to be expected. It must be concluded that the changes in polarization are due to a change in the relaxation time of the rotation. In turn, this cannot be due to a simple change in shape of the serum albumin neutral molecule, if this is an ellipsoid of axial ratio 5 or less. On changing into a sphere, such

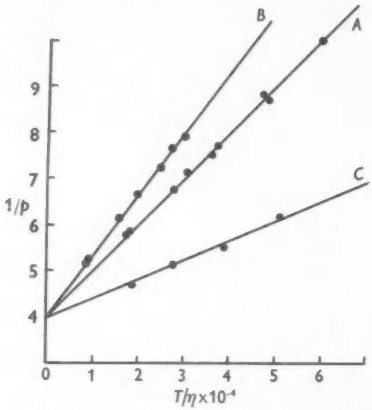

Fig. 9. *A*, polarizations of bovine serum albumin (0·12%) in salt-free pH 1·9 solution; *B*, 20% (w/v) sucrose at pH 1·9; *C*, solution *A* neutralized to pH 7.

an ellipsoid will have a relaxation time of 0·52 times the original value, while the observed ratio of β_0/β is 0·46. In order that this may be explained by a change in shape the neutral molecule would have to be an ellipsoid of elongation 8 or more or a flat molecule of axial ratio greater than 5. The absence of curvature in the plot of $1/p$ against T/η (Fig. 8) excludes the former, while the data of the dielectric dispersion (Oncley, 1942) do not support the latter. We may then conclude that molecular fission is the cause of the observed changes in the polarization. The number of resulting particles cannot be easily decided because of the unknown shape of the subunits. If these are less asymmetric than the parent molecule their number cannot be other than two, but if their asymmetry is greater a larger number is possible. The fact that the resulting particles may not all be of equal size should, within wide limits, have little influence on the observed slope, provided that the original and resulting particles have similar shape. If we assume that the groups that can react with the sulphonyl chloride have equal affinity for this reagent, the particles will contribute to the total fluorescent intensity in proportion to the number of such groups that they possess. In the absence of more detailed information it is convenient to assume that, in a solution of particles that have reacted together with the sulphonyl chloride, f_i the contribution of each species to the total emitted intensity is proportional to its relaxation time. Therefore

$$f_i = \frac{N_i \rho_{hi}}{\sum_i N_i \rho_{hi}},$$

where N_i is the number of molecules of relaxation time ρ_{hi}. The weighted harmonic mean of the relaxation times is

$$\bar{\rho}_h = \frac{1}{\sum_i \frac{f_i}{\rho_{hi}}} = \frac{\sum_i N_i \rho_{hi}}{\sum_i N_i} = \bar{\rho}_a,$$

where $\bar{\rho}_a$ is the number average of the relaxation times. Thus, if the original and resulting particles have the same shape, the ratio of the β quantities is the number of units into which the original molecule has dissociated.

The polarizations obtained at pH 2 in 20% (w/v) sucrose (Fig. 9) were systematically lower than in water at equal values of T/η. The sucrose points fall evenly on a straight line with $b = 1{\cdot}30$; $1/p_0 = 4$; $\beta = 2{\cdot}70$. The difference from the values in water may be due to more complete dissociation in sucrose (hydrogen bonds?) or to differences between the microscopic viscosity and the viscosity measured by flow. It may be pointed out that at pH 2 a small percentage of the sucrose present was hydrolysed and therefore a chemical reaction of the hexoses with the protein cannot be excluded.

Effect of salt on the acid dissociation. In the presence of M-potassium chloride, or 0·1 M-potassium sulphate the dissociation did not take place when the pH was brought from neutrality to 1·9. If the salt was added after the dissociation was obtained, complete reversal was observed, the polarization increasing to the original value in neutral solution.

Observations on bovine serum albumin in alkaline solution

0·5 ml. of 0·5% salt free conjugate was mixed with 2 ml. of 0·1 M-sodium hydroxide and the polarizations at different temperatures recorded. When measurements at temperatures higher than 25–30° were attempted an irreversible fall in the polarization was noticed. This was also noticed at lower temperatures if the alkali was left to act for a longer time (i.e. 12 hr. at room temperature). The polarization measurements below 28° were temperature reversible and could be repeated with several conjugates within 5%. As shown in Fig. 10, these measurements yielded $b = 1{\cdot}19$; $1/p_0 = 4{\cdot}34$; $\beta = 2{\cdot}56$. The polarization in 60% sucrose was 0·230 in excellent agreement with the extrapolated value.

Solutions left for an hour at 18°, and then neutralized, showed that the dissociation in alkali is

completely reversible as regards the relaxation time of the rotation. Solutions left for 10 hr. in alkali at 4° showed when neutralized a fall in the polarization indicating that if the action of the alkali is prolonged the reaction is no longer wholly reversible.

The slope and intercept of the solutions in alkali show differences from the values in acid. The comparatively minor differences in the values of β (0·46 and 0·40 respectively) can be due to a variety of causes, the analysis of which is not possible with the sole resources of this method. As regards the decrease in p_0 observed in alkali, several sources (Klotz, 1949; Laurence, 1952) indicate that the binding power of serum albumin is much diminished in alkaline solution. It may then be expected that

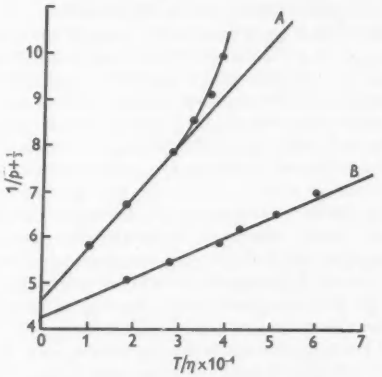

Fig. 10. *A*, polarizations of bovine serum albumin in 0·1 M-NaOH; *B*, a similar solution, kept for an hour at room temperature and then neutralized.

the freedom of rotation of the attached molecule should be larger in the latter case, leading to a decrease in the limiting polarization (Weber, 1952). The alkaline dissociation is largely unaffected by salt.

Effect of pH *on the relaxation time of the rotation*

Fig. 3 shows the effect of pH on the polarization of the fluorescence of 0·1% salt-free conjugates. The pH changes were obtained by addition of dilute hydrochloric acid or sodium hydroxide respectively and measured by glass electrode. The following points were noticed: (i) The shift in the polarization of the fluorescence with pH was complete in a matter of minutes, this being the time necessary to take a series of readings. In no case could a time effect be detected, even in solutions kept at 3°. (ii) Threefold dilution at any pH value did not result in any conspicuous change in the polarization. (iii) The main changes took place between pH 4 and 2·5 on the acid side, between 9·5 and 11·8 on the alkaline side. No further change was observed below pH 2 or above pH 12. (iv) The neutralized solutions showed no detectable difference from the original neutral solution. The most important fact to be noticed here is the complete correspondence of the regions of rapid change of the polarization with the main regions of titration of the protein.

Effect of urea treatment of the serum albumin conjugates

After treatment with 6 M-urea and subsequent removal of the latter by dialysis, the plot of $1/p$ against T/η, as shown in Fig. 11, gave a slope of

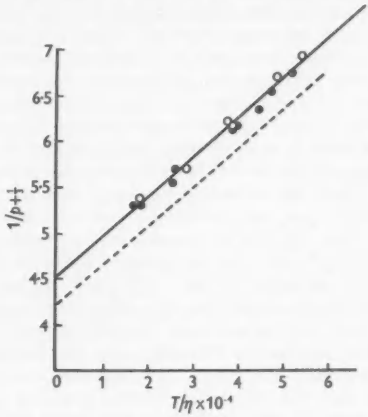

Fig. 11. Bovine serum albumin regenerated from 6 M-urea. The filled and open circles correspond to two independent experiments. The broken line is the slope of the native albumin.

0·40–0·42 in the different preparations, and intercept varying from 4·28 to 4·10. The slope was therefore not significantly different from that of the native protein, while the intercept was slightly, though consistently, higher. In 60% sucrose the observed polarization was $0{\cdot}245 \pm 0{\cdot}005$. The protein regenerated from urea has been found by sedimentation studies (Putnam, Erickson, Volkin & Neurath, 1943) to have a molecular weight equal to the native, and the observations here reported agree with it. The increase in the intercept can be interpreted as an increase in the rotational freedom of the coupled molecule due to decrease in the binding power of the protein, and a study of this property in urea-regenerated serum albumin may decide on the validity of this interpretation.

DISCUSSION

As described in the experiments, the sulphonyl chloride reacted very readily with the primary amino groups so that the formation of sulphon-

amido linkages as the most important product of the reaction with the protein offers little doubt. The absorption spectrum of the sulphonyl chloride presents a maximum at 369 mμ. and therefore, if any important amount of unreacted chloride were to remain adsorbed to the protein, a second maximum or an inflexion should appear in that region of the absorption spectrum of the conjugates. Such inflexion was observed in mixtures of proteins and chloride at the beginning of the reaction, but as this proceeded the inflexion decreased and disappeared.

Perrin's law of depolarization is followed by the serum albumin and ovalbumin conjugates within the limits of the experimental error. If the validity of Eqn. I, 28 is accepted, the absence of visible curvature may be taken as an indication that the molecules are flat or only moderately elongated. In all cases where the straight line law was followed excellent agreement was found between the limiting polarization p_0 as determined by extrapolation and the polarization observed in 60 % sucrose solution. The differences in the value of p_0 in the different conjugates may be attributed to the varying degree of rotation about its conjugating bond allowed to the coupled molecule. The calculation of absolute values of the mean relaxation time of the rotation requires τ_0 to be known. Relative values of ρ_h may, however, be calculated if τ_0 is assumed to be the same in the different conjugates. This can only be decided by direct measurement of τ_0, but the data presented show that the calculation of this quantity using the available figures for the relaxation times of the rotation of ovalbumin and serum albumin leads in both cases to the same value, namely $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec.

It is also assumed that no change takes place in the lifetime of the excited state between pH 1·5 and 14. Ovalbumin conjugates give the same polarization between these extreme values provided the observations in acid are done rapidly and at low temperature to avoid denaturation. The conjugates at pH 1–2 show a decrease in the fluorescence intensity and the lack of change in the lifetime of the excited state must be attributed to the long lifetime of the non-fluorescent form (Weber, 1948). A study of the quenching of the fluorescence of the sulphonamido derivatives of 1-dimethylaminonaphthalene-5-sulphonic acid makes clear that such is the case and that moderate degrees of quenching should not alter sensibly the value of τ_0. It is worthy of note that the quenching of the substituted sulphonamides took place at pH 3–4, while the conjugates showed little quenching (about 30 %) at pH 1·5. This difference is readily explained by the fact that at pH lower than the isoelectric point the ionic atmosphere of the protein will contain fewer hydrogen ions than the bulk of the solvent. The fluorescent intensity of the conjugates of serum albumin was considerably greater than those of native ovalbumin. Here once more the notion of a tautomeric non-fluorescent modification endowed with long life is probably valid.

Ovalbumin conjugates. The most important fact to emerge from the study of these conjugates is that reversible dissociation of the protein, by acid, alkali, or heat, was never seen and that the relaxation time increased considerably on denaturation by acid, alkali, heat, or urea. This increase in the observed relaxation time cannot be explained by an increase in the axial ratio of the particles alone. If the original quasi-spherical ovalbumin molecule is drawn into an ellipsoid of infinite axial ratio, Eqn. I, 28 shows that the apparent relaxation time cannot increase by a factor greater than 3. In the experiments, however, as shown in Figs. 4 and 5, increases of eight times and more were observed. These results lend themselves to a simple and complete interpretation if aggregation of denatured molecules is assumed to be the main cause of the increase in the relaxation time of the rotation. In the experiments on the time course of the acid denaturation, a visible precipitate appeared only in the case where the rate of increase of the relaxation time was a maximum, suggesting a continuous transition between the postulated aggregates and the precipitate. The isoelectric precipitation of denatured ovalbumin may be considered as the continuation of the aggregation on to a macroscopic scale.

If the aggregation is due to interactions which, like hydrogen bonds and van der Waals forces, decay rapidly with the distance, the main factor determining the growth of the particles will be the extent to which the electrostatic repulsion between the molecules can offset the thermal agitation and thus prevent the approach and interaction that leads to permanent binding (Verwey & Overbeek, 1948). The addition of electrolyte by decreasing the thickness of the double layer lets the particles grow larger and in the experiment at 30° the co-operative effect of salt and temperature allowed indefinite aggregation and flocculation to take place. The acid denaturation was accompanied by immediate increase in the relaxation time, while the protein denatured by alkali did not show it until the pH was brought near the isoelectric point. Moreover, when dissolved in acid, the alkaline denatured ovalbumin showed also increased relaxation time. A likely explanation of these facts is that the aggregation in acid is due to hydrogen bonds mainly from the uncharged carboxyl groups. No comparable source of hydrogen bonds exists in the alkaline protein.

The aggregation of ovalbumin in acid has been shown by Huang & Wu (1930) by osmotic pressure measurements.

Serum albumin conjugates. In contradistinction to ovalbumin the relaxation time of the rotation showed marked dependence on the pH of the solutions. The data presented show that the changes cannot be attributed to increase in τ_0 or to a simple change in shape of the serum albumin molecules, and that reversible dissociation into sub-units must be admitted. The study of this dissociation will be the object of another paper. It need only be indicated here that the departure from the linear law above 50° observed in neutral solution (Figs. 7 and 8) can be explained by thermal dissociation of the molecule.

SUMMARY

1. The preparation of stable fluorescent conjugates of ovalbumin and bovine serum albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride is described.

2. It is shown that the fluorescence of solutions of the purified conjugates follow Perrin's law of depolarization, yielding straight lines when the reciprocal of the polarization is plotted against the ratio of the absolute temperature to the viscosity of the solvent.

3. The slopes of the conjugates studied are reproducible within 2%, and for ovalbumin and bovine serum albumin at neutral pH are in the ratio of the harmonic mean of the principal relaxation times of the rotation as required by theory.

4. The polarizations observed in 60% sucrose solution are, in all cases studied, in excellent agreement with the limiting polarization obtained by extrapolation.

5. The relaxation time of the rotation of ovalbumin is shown to be independent of pH within a wide range (pH 1·5–14).

6. The denaturation of ovalbumin by acid, urea or heat results invariably in an increase in the relaxation time of the particles. The effect of temperature and of neutral salts on the acid denaturation has been studied.

7. The polarization of the fluorescence of bovine serum albumin conjugates is independent of pH between 4 and 9, but falls rapidly outside this region to reach lower stable values at pH 2 and 12 respectively. It is shown that only reversible dissociation into sub-units can explain these facts.

8. Bovine serum albumin regenerated from urea does not differ appreciably from the native species as regards particle size.

9. The preparation and characterization of 1-dimethylaminonaphthalene-5-sulphonyl chloride and of several sulphonamido derivatives are described.

The author is indebted to Dr Malcolm Dixon, F.R.S., under whose supervision this work was begun, for many suggestions and criticisms. He wishes also to express his gratitude to Dr K. Bailey and Dr F. Sanger, and to Mr D. J. R. Laurence and Mr T. C. Tsao for many interesting discussions. The micro-analyses have been performed by Weiler and Strauss, Oxford.

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A Study of the Adsorption of Dyes on Bovine Serum Albumin by the Method of Polarization of Fluorescence

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The ability of serum to bind small molecules was brought into prominence by the work of de Haan (1922) on the excretion of vital dyestuffs by the kidney. Grollman (1925) later showed by ultrafiltration that serum albumin possesses the property not possessed by ovalbumin, serum globulin, or gelatin, of adsorbing phenol red at alkaline pH. He showed that all the proteins studied bound the dye when the dye and the protein were of opposite electrical charge, but that serum albumin could also bind the negatively charged dye when it was itself negatively charged, adsorption decreasing rapidly, however, above pH 8. Bennhold (1932) showed that a variety of dyes and substances of pharmacological value was carried by the albumin fraction of serum and proposed for the protein a transporting function in the animal body. Recent studies on the binding by the serum albumin fraction (Smith & Smith, 1938; Rawson, 1943; Chow & McKee, 1945) and especially the detailed studies of Murray Luck and his co-workers on the stabilization of serum albumin (Boyer, Lum, Ballou, Luck & Rice, 1946) and the intensive physicochemical studies of Klotz (1949) have gone far to elucidate both the conditions under which adsorption takes place and the practical importance of the phenomenon (Edsall, 1947). Meanwhile, Scatchard, Scheinberg & Armstrong (1950) and Edsall, Edelhoch, Lontie & Morrison (1950) have shown that serum albumin also binds a number of negatively charged inorganic ions.

The present paper is concerned with a study similar in principle to those previously carried out, of the binding of certain fluorescent dyes to bovine serum albumin. The method used was that developed by Dr G. Weber in this department depending on a measurement of the polarization of the light emitted by excited fluorescent molecules (Weber, 1952).

THEORETICAL

The polarization of the fluorescent light from a dye molecule depends on the relaxation time of rotary Brownian movement of the dye in a given environment and on the time which the dye molecule takes to emit its light following an excitation. If the dye molecules are able to rotate extensively between excitation and emission, the fluorescent light has little overall polarization while if the molecules rotate very little in this time the emitting oscillators will remain appreciably orientated and will produce an overall polarization of the fluorescent light (Perrin, 1936). For the majority of dyes in aqueous solution the relaxation times of the molecules are small compared with the time taken to emit the fluorescence, and so the polarization of the fluorescence is small (usually less than 1 %). The relaxation time may be increased by placing the dye in a viscous medium such as glycerol (Weigert, 1920) or, as Weber has shown, by attaching the dye to a macromolecule such as a protein, which rotates more slowly in aqueous solution than does the free dye. The result of importance in the present study is that the fluorescence of the dye adsorbed on the protein is partially polarized, while the fluorescence of the free dye is usually unpolarized. Therefore, in any mixture of a fluorescent dye with a protein, where a part of the dye is bound to the protein and the remainder is free, the polarization P observed will be less than that for complete binding of the dye P_B depending on the relative contribution which the free and the bound dye make to the total fluorescent intensity. One can say that the polarized fluorescence of the bound dye is diluted by the unpolarized fluorescence of the free dye. A knowledge of the relative intensities of fluorescence of the dye, free and bound, and of the measured polarization of the dye-protein mixture enables the amount of dye bound to be easily calculated as follows. (For derivations of these formulae see Appendix.)

Assuming that the fluorescence of the free dye in solution is unpolarized, the ratio of P to P_B will vary between 0 and 1 as the degree of adsorption of the dye on to the protein is varied. Calling this ratio $\bar{p}$ and supposing the intensity of the fluorescence of the dye-protein system relative to that of the dye alone at the same concentration and pH is R it is shown that the fraction of dye bound (x) is

$$x = \bar{p}R - R + 1. \qquad (1)$$

If the dye does not change its fluorescent intensity on adsorption (i.e. $R = 1$) the simple formula

$$x = \bar{p} \qquad (1a)$$

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will hold. On the other hand, if the free dye in aqueous solution possesses a measurable polarization a second modification of Eqn. 1 is needed, namely that, where $\Pi = P_F/P_B$,

$$(1-\Pi)\,x = \overline{p}R - R + 1 - \Pi, \qquad (1b)$$

and P_F is the polarization of the free dye in solution.

Only one case was found in this work for which the simple formula (1*a*) could be used (see below); most of the dyes studied showed either a marked increase or a decrease in fluorescence when bound to bovine serum albumin. The significance of these changes in fluorescence will be discussed later, but it is enough to note here that if a marked increase in fluorescent yield occurs on adsorption (as in the case of the sulphonic acid derivatives of 1-naphthylamine), formula (1) ceased to be of practical use since the observed polarization is little diluted by the relatively poor fluorescence of the free dye. In these cases a formula

$$x = \frac{R-1}{R_{\max.}-1} \qquad (2)$$

has been used, in spite of the fact that this formula does not distinguish between a change in the fraction bound (x) and a redistribution of the already bound molecules among adsorption sites which affect the fluorescence to different degrees. A limitation of this sort is implicit in any indirect method such as this, especially where only one experimental variable is used, and may equally enter into the spectrophotometric technique (Klotz, 1946*b*). Since formula (1) includes two experimentally determined variables it is less likely to suffer from limitations of this sort and the formula has been used to detect one case (eosin) where the sites are of different kinds.

In order to determine the number of adsorption sites on the albumin and their affinity for the dye the equation derived by Klotz (1946*a*) was used, namely,

$$\frac{S}{Dx} = \frac{1}{n} + \frac{K}{nD(1-x)}. \qquad (3)$$

The known quantities in this equation are S and D, the total protein and dye concentrations respectively, and x, the fraction of dye bound, calculated as described above. The unknown quantities K, the equilibrium constant of dissociation of the eye-protein complex, and n the number of adsorbing sites, may easily be derived from the experimental curves, since n is the reciprocal of the intercept at the axis and K/n the slope of the graph. A greater slope means a more dissociable complex. When the dye concentration is small compared with the protein concentration the simple formula

$$nS\,\frac{(1-x)}{x} = K \qquad (3a)$$

will apply. This is a useful formula since, even if the adsorbing sites differ in combining power, the form of the equation remains the same.

EXPERIMENTAL

Dyes

The dyes used were chosen for their availability and possible experimental value. They can be classified chemically into three classes: (i) sulphonic acid derivatives of 1- and 2-naphthylamine containing one, two or three sulphonic acid groupings; (ii) derivatives of fluorescein and rhodamine with a xanthydrol ring system; (iii) derivatives of acridine. Where necessary these dyes were recrystallized or converted to crystalline derivatives and recovered. The purity of the dyes was examined by chromatography on paper. The term 'dye' has been used for these fluorescent compounds to simplify the nomenclature, although in some cases the absorption maximum of the compounds lies in the near ultraviolet region of the spectrum.

Serum albumin

The protein used was the Armour crystalline bovine serum albumin (batch no. 10522). It was found by comparison with the Armour fraction 5 albumin (batch no. 12 198) that the crystallization had not appreciably altered the adsorbing properties of the protein, but had removed some yellow material whose absorption of the exciting light might complicate the fluorimetric studies. The absorption at the excitation wavelength of 3600 A. was not great enough in the crystalline product to require any special corrections. The fluorescence of the crystalline protein was also unimportant with the yellow filters (Corning 338, 351, etc.) used for examining fluorescence. The protein was made up in 1% solution, dialysed for 24 hr. at 0° and filtered through Whatman no. 50 filter paper to give a clear solution. The protein concentration was measured in a Zeiss interferometer using the diffusate in the control compartment. The interferometer was calibrated using Kjeldahl nitrogen values for ovalbumin solutions.

Measurement of fluorescence

The protein and dye were mixed and a suitable buffer added. Experiments were made both at constant dye concentration and at constant protein concentration. When a series was carried out at constant dye concentration, only one comparison tube for free dye was required. When the dye concentration was varied, a comparison tube was made up for each dye concentration used. Observations were made in each case on the fluorescent intensity of the dye-protein mixture, as compared with the fluorescence of free dye; and on the polarization of the fluorescence of the dye-protein mixture. In cases where interest was found in the absorption spectrum of the mixture, this was measured in the Beckman spectrophotometer from 6000 A. down to 3100 A.

The limiting polarization P_B was measured by so increasing the protein concentration that no further increase in polarization resulted from addition of more protein. This condition was obtained with the dyes at certain pH values where binding was especially strong. The work of Weber (1952) suggests that the same value of P_B would be valid at other pH values.

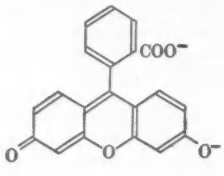
Since polarization measurements are most easily made visually, the intensity measurements were also made visually, the same filters for excitation and observation being used in both cases. Any changes in colour in the

fluorescence during adsorption would affect intensity measurements made with a photocell in an arbitrary way with respect to the eye and make the use of Eqn. 1 more difficult. Fluorescence of wavelength short enough to be readsorbed by the dye was excluded from observation by choice of filters. The visual fluorimeter was made by placing a Pulfrich photometer headpiece vertically at the end of an optical bench and splitting the beam of the usual Siemens mercury lamp, placed on the bench, by means of a modified Pulfrich beam-splitter. The cells containing the fluorescent solutions, size $4 \times 2 \times 1$ cm. were filled completely with the solutions and covered with pieces of optical glass. They were illuminated on their largest faces and observed through the top. The fluorescent intensities of a series under test were always compared with the brightest fluorescence of the series whether the free dye or the dye combined with the protein.

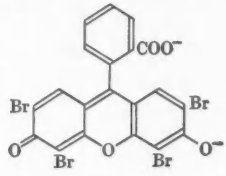
A simple correction was made to the intensity measurements, since the intensity observed from a solution producing polarized fluorescence is dependent on the polarization of the exciting light (Weber, 1952). In the measurement of polarizations the exciting light was polarized by a Nicol prism in a plane perpendicular to the axis of observation. In the fluorimeter, on the other hand, the exciting light was unpolarized. To make the two sets of measurements comparable the following formula (derived in the Appendix) was used where I' is the observed and I is the corrected intensity:

$$I = \frac{I'}{1 - \frac{1}{2}p}.$$

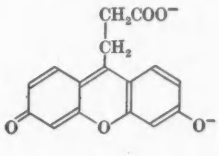
For the theoretical maximum of the polarization of 0·50 this correction is 33%.



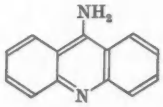
Fluorescein



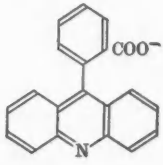
Eosin



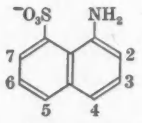
Succinylfluorescein



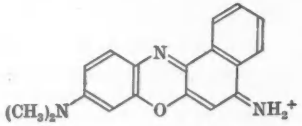
5-Aminoacridine



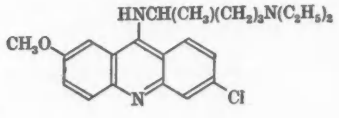
o-5-Acridylbenzoic acid



1-Naphthylamine-8-sulphonic acid



Cresyl fast violet



Mepacrine

Table 1. *Combination of fluorescein in borate buffer at* pH 9·1

(Fluorescein concentration = $0{\cdot}13 \times 10^{-5}$ mol./l.)

Protein concn. ($\times 10^5$ mol./l.) S	Polarization ($\times 100$) $100p$	Relative intensity R	$\bar{p}R$	Fraction of dye bound (x)	Equilibrium constant ($\times 10^5$) $K = nS(1-x)/x$*
1·46	7·6	0·61	0·13	0·52	1·35n
2·92	12·0	0·51	0·17	0·66	1·50n
5·84	17·0	0·37	0·17	0·80	1·45n

* Where n is the number of binding sites.

RESULTS

Table 1 gives some typical results of the combination of the bivalent negative ion of fluorescein with serum albumin in borate buffer at pH 9. The fluorescein concentration was small compared with the protein concentration and so Eqn. 3a applies.

The result of plotting according to Eqn. 3 is a straight line passing through the origin with slope determined by the equilibrium constant.

Table 2 illustrates a second simple case. This is that of the tervalent negative ion of the 2-naphthylamine-3:6:8-trisulphonic acid in unbuffered salt-free protein solution at pH 5·4. In this case there is strong binding to the protein so that over a range of concentration of both protein and dye two molecules of the dye are bound by one molecule of albumin (molecular weight 70 000). Extrapolation from Table 1 shows that fluorescein is only 0·26 times as fluorescent on the protein as in the solution. The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid, on the other hand, is but little altered by adsorption.

Table 2. *Combination of 2-naphthylamine-3:6:8-trisulphonic acid at the isolectric point*

Protein concn. $\times 10^5$ mol./l.	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
(a) Dye concentration $= 5 \times 10^{-5}$ mol./l.		
0·60	0·26	2·15
1·21	0·52	2·15
2·42	0·92	1·89

Dye concn. ($\times 10^5$ mol./l.)	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
(b) Protein concentration $= 1{\cdot}21 \times 10^{-5}$ mol./l.		
5·0	0·50	2·06
10·0	0·258	2·13
20·0	0·132	2·18

Table 3 gives data for the monovalent negative ion of 1-naphthylamine-8-sulphonic acid in phosphate buffer, pH 6·9. In this case the fluorescence of the dye is much increased on the protein (about

Table 3. *Combination of 1-naphthylamine-8-sulphonic acid at* pH 6·9

(Sulphonic acid concentration $4{\cdot}15 \times 10^{-5}$ mol./l.)

Protein concn. ($\times 10^5$ mol./l.)	Relative intensity ($\div 20$)*	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
0·00	0·05	0·00	—
0·725	0·37	0·34	1·95
1·45	0·62	0·60	1·72
2·90	0·84	0·84	1·19
7·25	1·00	1·00	—

* I.e. relative to the same concentration of free dye, allowing for the increase of fluorescence on adsorption.

20-fold), and the polarization rises to a maximum before the limit is reached in the increase of fluorescence. Using Eqn. 2 it appears that about 2 molecules of this substance are bound to the protein in the concentration range studied.

Effect of pH

As the pH in the surrounding solution is decreased and the charge of protein becomes more positive it would be expected that there would be an increase in the amount of a negative dye bound to

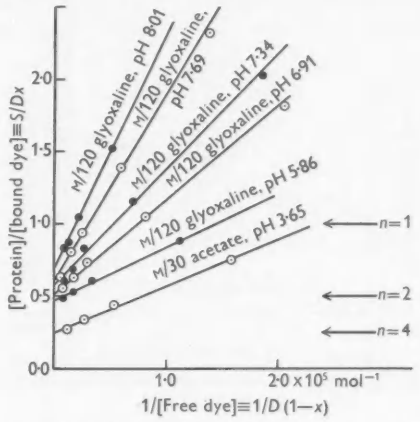


Fig. 1. Adsorption isotherms of 2-naphthylamine-3:6:8-trisulphonic acid.

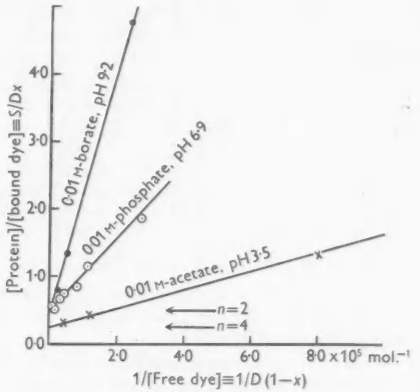


Fig. 2. Adsorption isotherms of fluorescein.

the protein. This is borne out by the following data for 2-naphthylamine-3:6:8-trisulphonic acid (Fig. 1), fluorescein (Fig. 2) and 1-naphthylamine-3:8-disulphonic acid (Fig. 3). It should be noted that fluorescein contains two titratable groups between pH 8 and 4, while the 1-naphthylamine-3:8-disulphonic acid contains one with pK 3·9. These reduce the negative charge on the dye at acid pH relative to alkaline pH.

Figs. 4 and 5 summarize the results obtained by plotting the polarizations obtained at an arbitrary protein concentration against the pH. Fig. 4 is for positive ions (basic dyes) and Fig. 5 is for negative ions (acidic dyes). The results illustrate the familiar observation that an opposite charge on the protein favours the combination of a dye, but there is also

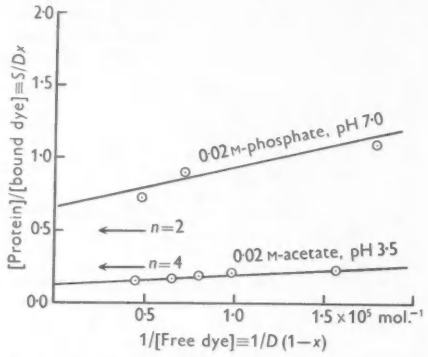


Fig. 3. Adsorption isotherms of 1-naphthylamine-3:8-disulphonic acid.

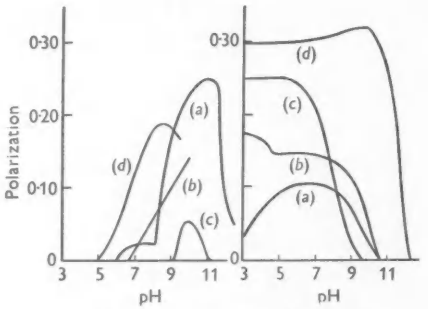


Fig. 4.

Fig. 5.

Fig. 4. Effect of pH on the adsorption of positive dyes (*a*) mepacrine, (*b*) cresyl fast violet, (*c*) 5-aminoacridine, (*d*) acridylbenzoic acid methyl ester.

Fig. 5. Effect of pH on the adsorption of negative dyes (*a*) fluorescein, (*b*) 1-naphthylamine-3:8-disulphonic acid, (*c*) 2-naphthylamine-3:6:8-trisulphonic acid, (*d*) eosin.

noticeable the special characteristic of serum albumin. This is apparent in both graphs. In Fig. 5 it is seen that negative ions, even tervalent ions, continue to combine with the protein on the alkaline side of the isoelectric point (pH 5·4). In Fig. 4, though the basic dyes combine in all cases with the protein, there are marked changes in extent of combination at the pK's of certain groups in the dyes. These changes lead to the conclusion that the neutral form of the dye (that form which is more lipid-soluble) is more strongly adsorbed than the positive ion, even though the average charge on the protein at these pH's would be expected to favour combination with the positive ion.

This is confirmed by the result of adding serum albumin to *o*-5-acridylbenzoic acid at pH 6 where the protein is slightly negative and the dye mostly in the zwitterion form with a negatively charged

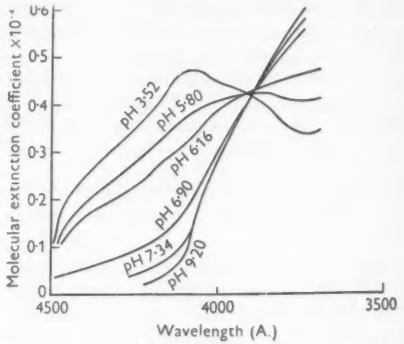


Fig. 6. Effect of pH on the absorption spectrum of *o*-5-acridylbenzoic acid.

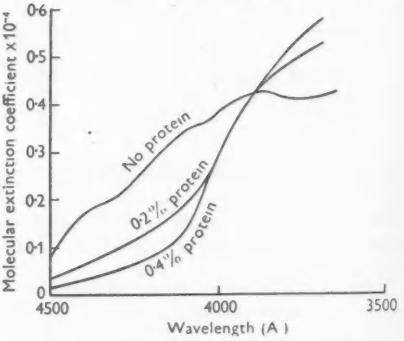


Fig. 7. Effect of the addition of serum albumin on the absorption spectrum of acridylbenzoic acid at pH 6·0.

carboxyl group and a positively charged heterocyclic ring. The serum albumin has the same effect on the dye as does a change of pH toward the alkaline side (Figs. 6 and 7). Addition of serum albumin to the dye at pH 9 when the heterocyclic nitrogen is uncharged has no effect on the spectrum. This 'reversed protein error' can be due to forces like those which produce the other effects discussed here. Fig. 8 shows adsorption isotherms for this dye.

That the neutral and ionic forms of the dyes differ markedly in the P_B values is made unlikely by experiments on the polarization of the fluorescence of the two forms in glycerol-water solutions of various viscosities. The conclusion follows that

while the presence of a charged group on the molecule is not a necessary condition for adsorption to occur, a positive group in contrast with a negative group has a strong inhibitory influence on the combination. This influence extends as far as pH 9·3, the pK of the ring nitrogen in 5-aminoacridine (Albert & Goldacre, 1943) when the protein has a net charge of about -30 (Tanford, 1950).

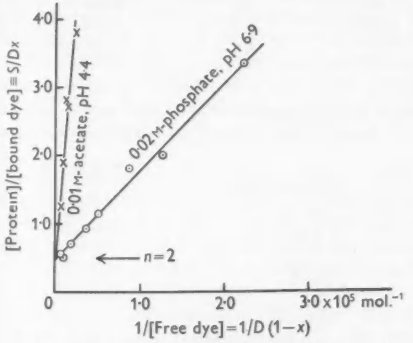


Fig. 8. Adsorption isotherms of acridylbenzoic acid.

Effect of modifications in structure of the dye

Some results of interest to the general theory of adsorption are obtained when the effect of minor alterations in the structure of the dye on its adsorption properties is studied. Fig. 9 shows results with fluorescein as compared with eosin in neutral phosphate buffer. Eosin is a case where the intensity does not change linearly with the calculated fraction bound (x) but first decreases and then increases as the protein concentration is increased. This unusual effect can be explained if only a few of the total binding sites yield an appreciably fluorescent adsorption complex and if it is assumed that these are the most actively combining sites. While the dye is in excess of the total sites, both the sites of low and of high fluorescent yield will be occupied. If an excess of sites is present, however, the sites of higher fluorescent yield will be favoured and the fluorescent intensity will increase again. There is therefore a minimum of fluorescence depending on the ratio of dye to protein. Using the formula 1*b* the amount bound is found to increase continuously over the range of protein concentration.

The naphthylaminesulphonic acids tried, namely the 1-naphthylamine-5- and 8-monosulphonic acids, the 3:8-disulphonic acid and the 3:6:8- and 4:6:8-trisulphonic acids and the 2-naphthylamine-3:6:8-sulphonic acid, showed binding of about the same extent at neutral pH. Methylation of the amino group increases the extent of combination of the 1-naphthylamine-5-sulphonic acid with serum albumin.

It can also be shown that fluorescein methyl ester and *o*-5-acridyl-benzoic acid methyl ester are adsorbed to about the same extent as the free acids. Sulphofluorescein is adsorbed similarly to fluorescein, and succinylfluorescein, with a short side chain instead of a ring, less than fluorescein.

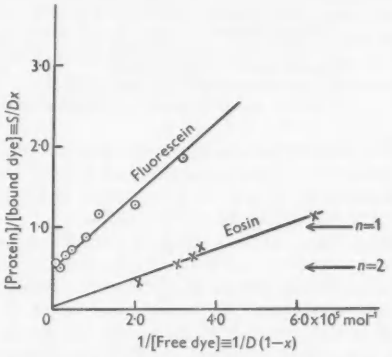


Fig. 9. Adsorption isotherms of eosin and fluorescein in 0·01 M-phosphate pH 6·9.

Competition experiments

As it has so far been described, the method of the polarization of fluorescence is limited to studying the combination of fluorescent substances only. A knowledge of the ability of various substances to compete with suitable test dyes leads, however, to an extension of the method in a more indirect form (Klotz, 1949). There are two easy ways of carrying out competition experiments:

(i) With a given quantity of serum albumin and of test dye the polarization P_1 is recorded and, in addition, the polarization P_2 (always less than P_1) which results when a known further amount of the same dye is added. If, instead of this further addition, an equimolar amount of a non-fluorescent competitor is added and a polarization P_3 results we have either

$P_3 = P_1$	The substance shows no competition
$P_3 = P_2$	Competes as strongly as the test substance
$P_1 > P_3 > P_2$	Competes less than the test substance
$P_3 < P_2$	Competes more than the test substance

This is illustrated by Table 4, where various derivatives of naphthalene are used to compete with the 2-naphthylamine-3:6:8-sulphonic acid. The sulphonic groups, but not the amino groups, seem necessary for competition. As the 1- and 2-naphthylamines themselves have no effect on the polarization, it seems likely that they do not combine at the site of combination of the test substance at the concentration used.

(ii) For substances whose combination with protein is fairly weak, competition can be studied by adding the competitor in great excess relative to the protein and noting the decrease in the polarization.

Table 4. *Competition of related compounds with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6·7)

Competitor	Amount of test substance (ml.)	Amount of competitor (ml. equimolar solution)	Polarization
None	1·0	—	0·060 P_3
None	0·2	—	0·116 P_1
1-Naphthylamine	0·2	0·8	0·115 P_3
2-Naphthylamine	0·2	0·8	0·117 P_3
Naphthalene-1-sulphonic acid	0·2	0·8	0·065 P_3
Naphthalene-2-sulphonic acid	0·2	0·8	0·052 P_3
Naphthalene-3:6:8-trisulphonic acid	0·2	0·8	0·035 P_3
N-Acetyl-1-naphthylamine-8-sulphonic acid	0·2	0·8	0·071 P_3

The present experiments have been confined to comparing the relative affinities of related simple substances as revealed by this method, although an absolute derivation should be possible.

Table 5 gives data for the ability of various ions to compete at the same molar concentrations, again using the 2-naphthylamine-3:6:8-trisulphonic acid as test substance. The outstanding trend in this table is that whereas alteration of the cation has little or no effect on the extent of the combination the ability of different anions to compete is markedly different. This agrees with the results found both in this study and by other workers that preferential combination occurs with the negative ion of an ionic compound in solution.

The competition with the series of univalent anions $F^- < Cl^- < Br^- < NO_3^- < I^-$ lies in their order in the so-called Hofmeister series relating to gelatin (Loeb, 1924) and this shows that the simple assumptions of the Debye-Hückel theory cannot in this case be sufficient to explain the effect of these salts. The effect of bromine atoms (see results with eosin above) and of nitro groups (Teresi & Luck, 1948) in organic molecules seem to be paralleled by the strong binding of the nitrate and bromide ions.

Table 6 gives a similar set of figures for some organic anions and one cation. The differences observed between the different compounds in this table are of importance for the theory of adsorption discussed below.

Table 5. *Competition of some ions with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6·7)

	Polarization
Competitor (M/15):	
None	0·202
NaCl	0·100
KCl	0·100
Na_2SO_4	0·015
K_2SO_4	0·015
Sodium acetate	0·130
Sodium succinate	0·015
Sodium benzoate	0·000
NH_4Cl	0·100
CsCl	0·101
NaF	0·181
KCl	0·098
KBr	0·050
KI	0·000
NaN_3	0·045
$NaNO_3$	0·015
Competitor (M/30):	
K_2SO_4	0·045
$(NH_4)_2SO_4$	0·047
Li_2SO_4	0·048
$MgSO_4$	0·070

The 2-naphthylamine-3:6:8-trisulphonic acid was used in these studies because its fluorescence intensity is little changed on adsorption and its

Table 6. *Competition of organic ions with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6·7)

Competitor	Competitor concn. (M × 10^{-2}) 0	1	1·5	3·5	6
	Polarization				
Sodium galacturonate	0·205	0·205	0·205	0·205	0·205
Sodium phenylacetate	0·205	0·162	0·102	0·000	0
Sodium *cyclo*hexylacetate	0·205	0·071	0	0	0
Sodium caprylate	0·205	0	0	0	0
Octylamine hydrochloride	0·205	0·205	0·205	0·148	0·095*

	Competitor concn. (M × 10^{-3}) 0	1	1·5	2·5
	Polarization			
Sodium caprylate	0·208	0·140	0·067	.0

* Attributable to the chloride ion alone.

polarization has, therefore, a linear relationship to the fraction which is bound. The next section considers more generally the changes in fluorescent properties and absorption spectra of the dyes.

Change in fluorescence andabsorption spectra on adsorption

In the previous sections the change in fluorescent intensity on adsorption has been mentioned as an important factor to be considered in applying the method of the polarization of fluorescence to a study of adsorption isotherms. During adsorption there are also changes in the absorption spectra of the dyes. The changes may be summarized conveniently according to the chemical types of the dye molecules.

(i) *Sulphonic acid derivatives of 1- and 2-naphthylamine.* The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid is bright sky blue and is slightly quenched when the dye is on the protein without a change in colour. The fluorescences of the 1-naphthylamine derivatives are greenish yellow in aqueous solution and this is also the case with the dimethylaminonaphthalene derivative. The fluorescence of all these compounds is increased when they are adsorbed on the protein and in most cases it is changed to the bright blue of the 2-naphthylamine compound described above. This change is most marked with the relatively weak fluorescence of the 1-naphthylamine-8-sulphonic acid where a 20-fold increase in brightness occurs. With the stronger fluorescence of the others in this series, increases of two or fourfold are observed. The change may be due to an increase in the dipole moment of the amino group on adsorption, as it is known that 2-naphthylamine possesses a higher dipole moment than 1-naphthylamine (Bergmann & Weizmann, 1936). Shifts to the red of 80A. were observed in the ultraviolet spectra of the 1-naphthylamine compounds when adsorbed.

(ii) *Xanthydrol derivatives.* The fluoresceins and rhodamines tried were all quenched on the protein. For fluorescein itself the intensity was 0·26 of that of the free dye. In addition, characteristic shifts in the absorption spectrum towards the red occurred. This effect was most marked with the methyl ester of fluorescein where the sharp absorption bands of the free and of the adsorbed dyes are clearly separated at the maxima, while the shape and height of the curves are hardly altered (Fig. 10). The colours of solutions of the adsorbed methyl ester are bright pink while that of the free dye at alkaline pH is orange yellow. This is not an effect of pH, as the absorption of the different ionic forms of fluorescein moves progressively towards the blue with increased acidity. In the case of eosin the adsorbed dye has a moderate adsorption at the green line of mercury, namely, 5460A. while the free dye absorbs little at this wavelength. The tubes which contain adsorbed material can therefore be distinguished easily as they are brightly fluorescent in this light, whereas tubes with the free dye alone are by comparison practically non-fluorescent.

(iii) *Acridine derivatives.* Considerable variation was found in the behaviour of the fluorescences of the acridines when adsorbed. The fluorescence of *o*-5-acridylbenzoic acid was quenched to one-tenth of its value in solution without change in its absorption spectrum. The fluorescence of mepacrine increased slightly.

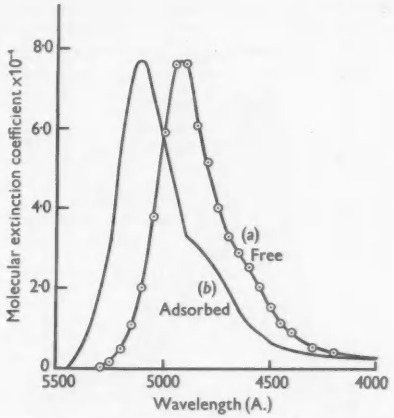


Fig. 10. Absorption spectrum of fluorescein methyl ester at pH 9 (*a*) free dye, (*b*) dye adsorbed on serum albumin or cetyltrimethylammonium bromide.

The relation of the changes in fluorescences and absorption spectra to the state of the environment of the dyes

Since the change in the fluorescences and the absorption spectra of the dyes is likely to be characteristic of some new electronic state due to an environment at the site of adsorption different from that in water, it was considered likely that a simulation of these changes in simple physico-chemical systems would lead to an understanding of the nature of the adsorbing region on the protein. A suitable series of dyes was chosen to test this, namely, eosin, fluorescein methyl ester, 1-dimethylaminonaphthalene-5-sulphonic acid, 1-naphthylamine-8-sulphonic acid and *o*-5-acridylbenzoic acid. By trial it was found that the katonic detergent, cetyltrimethylammonium bromide (CTAB), gave reactions with these dyes very similar to those given by bovine serum albumin. That adsorption by the detergent micelles had occurred was demonstrated by showing that the detergent in low concentration increased the polarization of eosin excited with the

green line of the mercury lamp and of fluorescein excited with the blue lines. Klotz (1947), using the azo-dye methyl orange, has shown a similar parallel between the effect of protein and of CTAB on the absorption spectrum. The ring structure of the dye which acts as the oscillator (Benel, Kastler & Rousset, 1940) is likely to be in the micelle rather than adsorbed on its surface (Hartley, 1937), and hence in a substantially organic environment free from water dipoles.

An important property of the interior of the micelle is its low dielectric constant, and in a single-phase system the dielectric constant of the solutions of the dyes was lowered by adding dioxan (Akerlof & Oliver, 1936) which had been purified by the

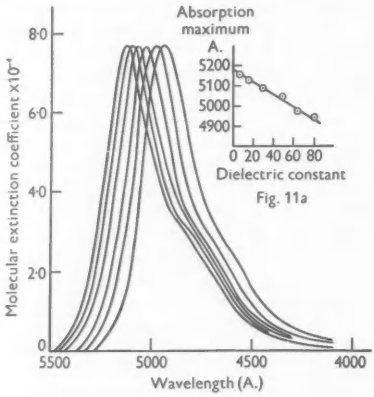


Fig. 11. Effect of dioxan concentration on the absorption spectrum of fluorescein methyl ester (from right to left, increasing dioxan concentrations from 0 to 90%.)

Fig. 11*a*. Shift in the wavelength of the absorption maximum with increasing dielectric constant of the dioxan-water mixtures.

method of Oxford (1934). The dyes behaved in a way similar to that observed when they were adsorbed by serum albumin and CTAB, the most striking similarities being shown by fluorescein methyl ester, 1-naphthylamine-8-sulphonic acid and acridylbenzoic acid (Figs. 10–13). The effect of solvents of low dielectric constant in imitating the effect of serum albumin is such that we could almost without exception predict how a given dye would change its fluorescence and spectrum from its behaviour in dioxan solution. Evidence that the dielectric constant of the medium is the determining factor in the changes in spectrum of the xanthydrol compounds may also be obtained from the results of Vaillant (1927) for erythrosin, though this author does not interpret his results in this way. He found that the largest shifts to the red in the spectrum of erythrosin were in benzene and 'essence', both solvents of non-polar nature. Glycerol, concentrated sugar solution and ethanol gave smaller shifts to the red with respect to water, although the refractive indices and hence the volume-polarizabilities of these liquids was as high or higher than the benzene and 'essence'. A similar view is admitted by Seshan (1936).

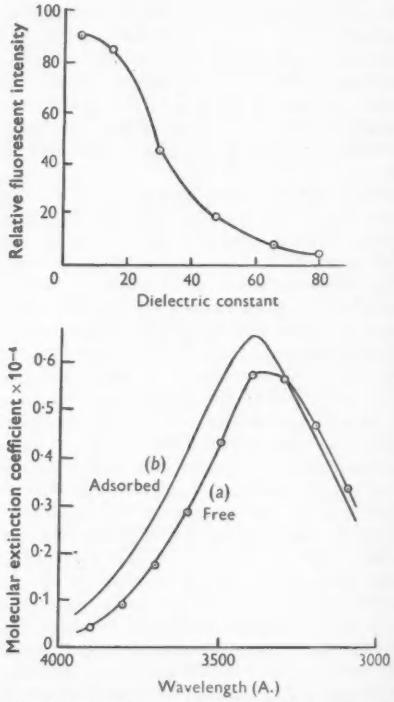


Fig. 12. Effect of dioxan on the absorption spectrum and fluorescent intensity of 1-naphthylamine-8-sulphonic acid. (*a*) spectrum of dye in aqueous solution, (*b*) spectrum of dye adsorbed on serum albumin or in 90% dioxan solution.

It may be concluded that the behaviour of the fluorescent dyes when adsorbed on to serum albumin identifies the site of adsorption with material of low dielectric constant. The use of a series of these dyes should enable other systems of amphipathic behaviour to be easily distinguished as has already been stated (Sheppard & Geddes, 1945).

Relation to the work of Dr G. Weber with fluorescent groups coupled by chemical reaction with proteins

Of the dyes used in this paper, two, namely the 2-naphthylamine-3:6:8-trisulphonic acid and the 1-dimethylaminonaphthalene-5-sulphonic acid, have

been used by Dr G. Weber as compounds suitable for coupling with proteins by covalent bonding. It is possible to carry out studies using serum albumin similar to those of Weber (1952) by ensuring that all the dye is combined with protein and plotting a curve of $1/P_B$ against T/η, where T is the absolute

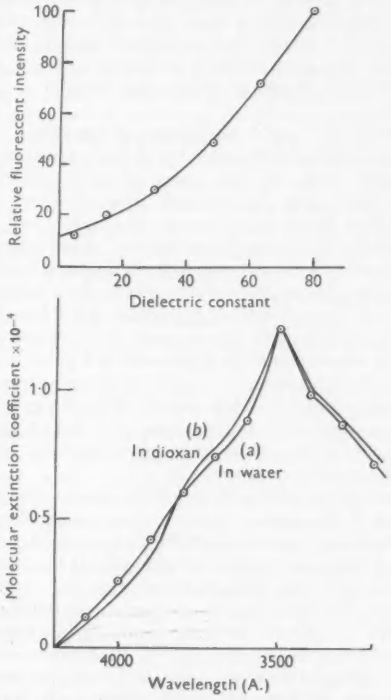


Fig. 13. Effect of dioxan on the absorption spectrum and fluorescent intensity of *o*-5-acridylbenzoic acid. (*a*) spectrum of dye in aqueous solution, (*b*) spectrum of dye in 90% dioxan.

temperature and η the viscosity. The intercept of this line with the ordinate gives the value of $1/P_B$ in the absence of Brownian movement, and the slope is determined by the lifetime of the excited state and by the size of the protein. In either case the value of $1/P_B$ was found to be the same at $T/\eta = 0$ for the coupled as for the adsorbed dye. The slope of the graph for the adsorbed 1-dimethylaminonaphthalene-5-sulphonic acid was found to be twice that for the coupled sulphonamide conjugate (Fig. 14). Since Weber (unpublished observations) has shown that the lifetime of excited state of the acid in glycerol is about twice that of the derived sulphonamide, these results show that the adsorbed dye has a fixity in the albumin surface comparable with that of the coupled dye. A similar result is obtained for eosin excited by Hg 5460A. where an extrapolation of $1/P_B$ to $T/\eta = 0$ gives the accepted value for eosin in very viscous solution. The colour of the fluorescence of the serum albumin-sulphonamide conjugate is greenish blue like the colour of fluorescence of the simple sulphonamides in 90% dioxan. Digestion of the albumin conjugate with Armour crystalline pepsin results in the fluorescence becoming orange like that of the sulphonamides in water. Even with native proteins the greenish-blue fluorescence of albumin conjugates is not universal (Weber, 1952) and the yellowing of the fluorescence of the sulphonamides as the polarity of the medium is increased may provide a convenient yardstick against which the polarity of the protein surface in the neighbourhood of the primary amino groups may be estimated.

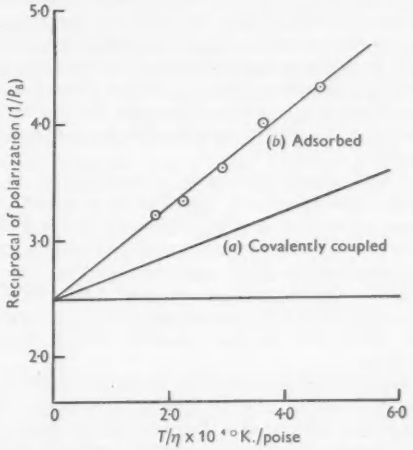


Fig. 14. Plot of polarization at different temperatures according to the Perrin equation for the 1-dimethylaminonaphthalene-5-sulphonic acid attached to serum albumin (*a*) covalently coupled (Weber's data), (*b*) adsorbed.

DISCUSSION

The evidence presented here and that obtained by other workers leads to the view that there are several kinds of physicochemical mechanism operating in the process of adsorption on to serum albumin.

It is clear that the charge on the molecule is of importance, negative ions being adsorbed in preference to positive ions at the sites of adsorption. Superimposed on this is a variation in binding depending on the nature of the ion. It is often supposed that this effect is due to van der Waals forces between the protein and the adsorbed molecule. Since, however, for compounds of about the same molecular weight, namely, caprylic acid, *cyclo*hexyl

acetic acid and phenyl acetic acid, one obtains different degrees of binding with the benzene moiety less active than the paraffin moieties, this does not seem to be an adequate hypothesis.

Any organic molecule may be considered as an assembly of polar and of non-polar parts, and in aqueous solution the relation of these parts with the water will be very different. Non-polar material introduced into water produces a separation of the strongly bonded water molecules and replaces the interaction of water molecules with each other by a weaker interaction of water with the non-polar substance. Reversal of this process with recombination of the separated molecules of water results in a liberation of energy which corresponds to the energy of the surface of the non-polar material in solution. The polar parts of the molecule, in contrast, can associate with the water as well as can water with itself (Langmuir, 1925) and the adsorption of the non-polar part of the organic molecule on to or into a non-polar surface, leaving the polar parts in the aqueous phase, is therefore accompanied by a liberation of energy, since the surface of the non-polar material in contact with the water is decreased.

A semi-quantitative way of stating this is to assign to water a dipole W and to the so-called non-polar material a smaller dipole P (Gent, 1948). Before adsorption the dipole interaction energy is proportional to $2PW$ and after adsorption to P^2+W^2. The change in energy is P^2+W^2-2PW or $(W-P)^2$ which increases as the difference between the respective dipoles increases and is always positive.

From the behaviour of the fluorescent dyes when adsorbed there is good reason to suppose that adsorption does remove the dye from the water to a second medium of lower dielectric constant, and so it seems plausible to attribute the adsorption in great part to a rejection from the water structure rather than an attraction by the protein. The concept of surface energy further accounts for the relatively low activity of a benzene moiety as compared with a paraffin, since the surface energy of benzene in water is less (34 ergs/cm.^{2}) than for a paraffin (54 ergs/cm.^{2}); benzene is more hydrophilic than paraffin. As would be expected, galacturonic acid, although its molecular weight is higher than most of the simple competitors included in Table 6, is ineffective in competition, as it is strongly hydrophilic and the force of rejection is weak.

In terms of the serum albumin molecule the amino-acid composition of the protein is adequate to provide the sort of surface against which these forces can act. It is visualized that the region of adsorption is one where a local excess of positive groupings is backed by a local excess of the hydrophobic side chains of leucine, valine and other non-polar amino-acid residues. Because of the low dielectric constant of this microphase the local charge is shielded from the general field of the protein and is not as greatly affected as it would otherwise be by variations in the net charge of the protein as a whole (Hartley, 1938). To account for the absence of corresponding sites of adsorption for positive ions, it must be supposed that the negative groups on the protein are more diffusely arranged and lack the hydrophobic backing which the positive groups have.

For each molecule the extent of adsorption will depend both on non-polar forces and attraction of negative ions. In the series of naphthylaminesulphonic acids, for example, the addition of a sulphonic group will decrease the former and increase the latter type of interaction, the effect near the isoelectric point being to keep the total binding of these acids appreciably constant. An additional type of force may be important with bromine, iodine and the nitro group, both in inorganic ions and in organic binding. This could be a polarization in the field between the protein and the orientated dipoles of water. The low activity of the fluoride ion, as compared with the chloride, may be due to its increased hydration, keeping it in the aqueous phase by means of hydrogen bonding.

In a recent review Klotz (1949) has described his views on the special structure of the serum albumin molecule and has criticized the ideas of Davis (1943). The present work supports and extends the ideas of Davis and is in disagreement with the views of Klotz, as it underlines the importance of the surface active properties of those amino-acids residues usually regarded as inert, namely, the leucines, valine, etc. Klotz, in contrast, believes the stoicheiometric relations of hydroxy amino-acids with the dicarboxylic acids to be of paramount importance. The correlations given by Klotz are nevertheless suggestive, as it is for those proteins where the hydroxyl groups are relatively less numerous that the hydrophobic backing of charged groups would be most easily achieved and the forces described in this paper most likely to be active. In view of the non-polar nature of the active surfaces on the protein and of the adsorbed molecule the idea of 'combined' water molecules being displaced on adsorption (Klotz, 1949) would seem unlikely.

The results with the dyes used in this work show that about 2–4 molecules of dye are adsorbed by each molecule of the protein at neutral pH, with the exception of eosin where more molecules combine. A decrease in the pH increases the number of sites available to negative dyes. Rosenfeld & Surgenor (1950) have found 2 molecules of ferriprotoporphyrin strongly bound to the human serum albumin molecule, while Martin (1949) has found 3 molecules

of bilirubin bound per molecule of albumin. These results contrast with the much higher values from the dialysis experiments of Klotz (1946*a*) and other workers, e.g. Teresi & Luck (1948) (10–25 molecules bound per albumin molecule). Klotz (1946*b*) has never reported these high values as a result of his spectrophotometric technique, and it may be that the dialysis technique when applied to higher dye concentration includes some type of interaction between the dye and the protein unlike that occurring at lower concentrations and measured by the present method. Certainly the low values are more in accord with the view that the effect of the positive groups is reinforced as described in this paper in a few special patches on the protein, while the higher values accord better with the view of Klotz that the amino groups need only to be freed from hydrogen bonding with other amino-acid residues in order to be active. The results of a comparison with Weber's work on covalently coupled dyes, discussed above, strongly support the idea of a special patch of positive groupings as opposed to a general activation.

Whether a similar active region is important in cases of interactions of a more specific nature requires further evidence to decide. It is likely that the forces related to surface tension described here play a part in all interactions of non-polar material in aqueous solution, and the forces of van der Waals should therefore not be used indiscriminately to account for these interactions. The analogy between the electronic state of the dye molecules at the protein surface and in organic media should be borne in mind when attempting to formulate a theory of enzyme activation.

SUMMARY

1. A method is described for studying quantitatively the adsorption of fluorescent dyes by macromolecules using measurements of the polarization and intensity of the fluorescent light. The method is applied to the adsorption of dyes by bovine serum albumin.

2. The effect of pH on the adsorption is discussed. Negatively charged dye ions are adsorbed even when the net charge on the protein is negative. A positive charge on the dye molecule inhibits combination on either side of the isoelectric point. Basic dyes are adsorbed as the free base but not as the positive ion.

3. Free carboxyl, sulphonic, hydroxyl or amino groups in the dye are not necessary for combination between dye and protein to occur. Bromine atoms increase the extent of adsorption.

4. Addition of related but non-fluorescent compounds can decrease the adsorption of a fluorescent dye by competition. Competition is also found between the dye and a variety of simple inorganic and organic ions. The activity of univalent negative inorganic ions is in the order of the Hofmeister series and that of univalent negative organic ions in the order of surface activity. Positive ions do not compete with the dyes.

5. Characteristic changes in the absorption spectra and in the fluorescence of the dyes occur on adsorption. These changes can be reproduced if the dyes are adsorbed by cetyltrimethylammonium bromide micelles or dissolved in 90 % dioxan.

6. The significance of these results is discussed and a comparison made between the adsorbed dye and the dye coupled covalently to serum albumin by the methods of Dr G. Weber. It is concluded that the results of this work can best be explained by the presence in serum albumin of a few regions of surface where a local excess of positively charged amino-acid residues is backed by residues with non-polar side chains.

I am indebted to Dr G. Weber of this laboratory for initiating this work, for critical advice during its progress, and for making available the apparatus for measurement of polarization of fluorescence and many of the organic chemicals used. I would also like to thank Dr M. R. J. Sultan of the Department of Colloid Science, Cambridge, for providing the cetyltrimethylammonium bromide, Dr F. A. Isherwood of the Low Temperature Research Station, Cambridge, for a gift of purified galacturonic acid and Messrs Imperial Chemical Industries Ltd. for the naphthylaminesulphonic acids. The work was carried out during the tenure of a grant from the Medical Research Council.

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APPENDIX

Derivation of equations

Formulae (1) are derived as follows: consider a solution of dye with fluorescent intensity I_0 and polarization P_F to which protein is added. As the concentration of protein increases the fraction of dye bound to the protein increases. A small addition of protein will remove an amount of dye δx, of fluorescent intensity $I_0 \delta x$, from solution. Attached to the protein this amount of dye will have a different intensity $\lambda_x I_0 \delta x$, say, where λ_x may vary during the course of a large addition of protein. It will also have a polarization P_B larger than P_F. When a fraction x of the dye is bound the intensity of the mixture I will be

$$I = I_0 - \int_0^x I_0 \mathrm{d}x + \int_0^x \lambda_x I_0 \mathrm{d}x$$

$$= I_0 \left(1 - x + \int_0^x \lambda_x \mathrm{d}x\right). \qquad \text{(i)}$$

The polarization p is the average for free and bound dye weighted according to their respective intensities, i.e.

$$p = \frac{(1-x)\,P_F + \int_0^x P_B \lambda_x \mathrm{d}x}{1 - x + \int_0^x \lambda_x \mathrm{d}x}. \qquad \text{(ii)}$$

With P_B not dependent on x, the integral $\int_0^x \lambda_x \mathrm{d}x$ is eliminated from (ii) using (i). Calling

$$P/P_B = \overline{p}, \quad P_F/P_B = \Pi, \quad I/I_0 = R;$$

$$\overline{p} = \frac{(1-x)\,\Pi + R + x - 1}{R}, \qquad \text{(iii)}$$

or

$$(1-\Pi)\,x = \overline{p}R - R + 1 - \Pi. \qquad (1b)$$

Equations (1) and (1*a*) are derived from this by putting $P_F = \Pi = 0$ and $R = 1$ progressively.

Formula (2) is based on a simple proportionality argument. Formula (4) is derived as follows: Suppose the fluorescence is excited by light plane polarized at an angle θ to the axis of observation and has a polarization p. The observed intensity I' will be the sum of three components, namely,

$$I' = I_1 \sin^2\theta + I_2 \cos^2\theta + I_2, \qquad \text{(iv)}$$

where I_1 and I_2 are the intensities of the components of fluorescence parallel and perpendicular to the exciting vector and so $p = \dfrac{I_1 - I_2}{I_1 + I_2}$ by definition, or $I_2 = \dfrac{(1-p)}{(1+p)}\, I_1$. Unpolarized excitation is equivalent to averaging for all angles θ or since $\overline{\sin^2\theta} = \overline{\cos^2\theta} = \frac{1}{2}$;

$$I' = \tfrac{1}{2} I_1 + \tfrac{3}{2} I_2$$

$$= I_1 \left(\frac{1}{2} + \frac{3}{2}\frac{1-p}{1+p}\right)$$

$$= I_1 \left(\frac{2-p}{1+p}\right). \qquad \text{(v)}$$

For excitation perpendicular to the direction of observation on the other hand $\theta = 90°$, and

$$I = I_1 + I_2$$

$$= I_1 \left(1 + \frac{1-p}{1+p}\right)$$

$$= I_1 \left(\frac{2}{1+p}\right). \qquad \text{(vi)}$$

Combining (v) and (vi)

$$I = \frac{I'}{1 - \frac{1}{2}p}. \qquad (4)$$

Enzymes of the Potato Concerned in the Synthesis of Starch

1. THE SEPARATION AND CRYSTALLIZATION OF *Q*-ENZYME

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Following an early recognition that plant starches are not homogeneous, extensive work in the last decade has established that most plant starches contain two main components. The isolation and characterization of the components is reviewed fully elsewhere (Kerr, 1950) and need not be described here. It is sufficient to say that these components, for which the names amylose and amylopectin are becoming commonly accepted, are readily separated from each other by fractional precipitation from aqueous solution with organic reagents, and that this difference in solubility and in other physical properties is due not to any considerable difference in chemical composition, but to a difference in molecular shape. Thus both components are polymers of glucose of high molecular weight, but amylose has a linear, or at most very slightly branched structure, while amylopectin has a very considerably branched structure.

Amylose is composed essentially of a single chain of glucose units joined by α-1:4 bonds. The exact structure of amylopectin is not known, but the available evidence (Halsall, Hirst, Hough & Jones, 1949) is consistent with the view of Haworth, Hirst & Isherwood (1937) of a main chain of glucose units linked as in amylose by α-1:4 bonds, but with an average of about every twelfth such bond replaced by an α-1:6 bond. At each of these positions a side chain of about twelve α-1:4 linked glucose units is attached at its reducing end by an α-1:4 bond. Half of the amylopectin molecule is therefore in the form of short side chains.

Along with the elucidation of the composition of starch, some insight was gained into the biological origin of its components: amylose was shown by Hanes (1940*a*, *b*) to be formed from glucose-1-phosphate by the action of the plant enzyme phosphorylase; amylopectin was shown by Haworth, Peat & Bourne (1944) to be formed from amylose by the plant enzyme, *Q*-enzyme.

The synthetic polysaccharides prepared in the laboratory with these enzymes are likely to prove of assistance in the study of natural starch, and, in addition, they can serve as model substances for the study of the effect of degree of branching on the properties of polymers. Such studies require the provision of pure enzymes, and it was the purpose of the present work to obtain phosphorylase and *Q*-enzyme of a sufficient degree of purity.

Part of the method adopted was based on the technique devised at the Harvard Medical School, Boston, for the fractionation of blood plasma. In this technique precipitation is carried out at low temperature, with ethanol, from solutions of sufficiently low ionic strength for the specific properties of the proteins to exert their full effect (Cohn *et al.* 1946; Cohn *et al.* 1950). The separation of *Q*-enzyme by these methods, reported briefly earlier (Gilbert & Patrick, 1950), is described in this paper, and the separation of phosphorylase is described in the following paper.

METHODS

Preparation of crystalline Q-enzyme. Autumn harvested, non-sprouting King Edward potatoes were peeled carefully, washed with tap water and sliced. The slices (400 g.), after steeping for 0·5 hr. in 500 ml. of $Na_2S_2O_4$ solution (7 g./l.) were drained, washed thoroughly with tap water, and minced. The juice was pressed from the pulp through fine muslin by a hand press, and clarified by centrifuging. About 100 ml. were usually obtained. Substitution of a blender (at 0°) for a mincer improves the yield of juice to about 200 ml. From this point all operations were carried out in a cold room set at −5°, or in a refrigerated centrifuge. A 50% (v/v) solution of ethanol containing 0·01 M-disodium hydrogen citrate was prepared for use as a precipitant (50 ml. ethanol, 53·7 ml. water). Its pH was adjusted with NaOH until a pH of 6·0 at 20° was recorded for a portion diluted with 5 times its volume of 0·01 M-NaCl. The precipitant was cooled to −5°, and added at a maximum rate of 25 ml./min. with thorough but gentle stirring. A further 15 min. were allowed for equilibration before centrifuging. The temperature of the solution to which the precipitant was being added was kept about 1° above its freezing point by a refrigerator bath of ethanol.

The potato juice (100 ml., pH 5·8–6·0) was cooled to 0°, avoiding freezing, and 28·2 ml. of 50% ethanol added to bring the concentration of ethanol to 11% (at a final temperature of about −2°). After centrifuging the solution at −2° the precipitate was discarded and the ethanol concentration of the supernatant raised to 14·9% by adding 0·11 ml. of 50% ethanol/ml. of supernatant to give precipitate I. (The figure 16 instead of 14·9 was given in error by Gilbert & Patrick (1950).) The precipitate was separated by centrifugation at −5°, dissolved at 0° in 60 ml. of citrate

buffer (0·01 M-disodium hydrogen citrate, NaOH to pH 6·0), and 16 ml. of 50% ethanol added to give a concentration of 10·5% (v/v). The resulting precipitate was discarded, and the concentration of ethanol raised to 15% by the addition of 0·11 ml. of 50% ethanol/ml. of supernatant as before. Precipitate II which formed was dissolved at 0° in 40 ml. of 0·01 M-citrate solution, pH 6·0, and reprecipitated at −5° with 18·9 ml. of 50% ethanol to give precipitate III. This was dissolved at 0° in 5 ml. 0·05 M-citrate buffer pH 7·0 and the solution freeze-dried to enable the experiment to be interrupted at this point. The dried material was later reconstituted with 20 ml. water and the ionic strength of the solution increased 3·6 units by the addition of 9·3 ml. of 50% (w/v) $(NH_4)_2SO_4$ solution (50 g./100 ml. solution, NH_3 to pH 6·2). After standing for 2 hr. at 0° with frequent stirring, the solution was centrifuged at 0° and precipitate IV removed.

Precipitate IV was dissolved at 0° in 10 ml. of $(NH_4)_2SO_4$ solution (ionic strength 1·0); (4·4 g./100 ml., NH_3 to pH 6·2), and the solution filtered first through a Whatman no. 42 paper under gravity, and then through a sintered-glass filter (grade 3) under slight positive pressure. After raising the temperature to 10°, 50% (w/v) $(NH_4)_2SO_4$ solution pH 6·2 (filtered through a glass filter, grade 3) was added drop by drop until a faint precipitate appeared (about 5 ml. were required), after which the precipitate was just redissolved by the addition of a few drops of water. The solution was then cooled to 0°, and left overnight to crystallize in a stoppered bottle, which it almost filled to leave a minimum of air-liquid surface. The crystals, V, were centrifuged at 0°. Recrystallization was carried out by dissolving the crystals in $(NH_4)_2SO_4$ solution, as for precipitate IV, and repeating the operation above.

Analytical methods. Protein N was determined by the method of Falconer & Taylor (1946). A conversion factor of 6·25 was adopted arbitrarily to calculate protein.

The action of *Q*-enzyme on amylose or starch can be followed by sampling and staining with iodine solution. The stain becomes progressively less blue as the reaction proceeds (Bourne, Macey & Peat, 1945), and finally takes on the hue characteristic of amylopectin. This colour change can be used for the quantitative estimation of *Q*-enzyme and for a determination of activity/unit weight by applying an empirical relation described earlier (Gilbert & Swallow, 1949) for the rate of decrease of optical density at 6800 A. It was found then that if a non-retrograding 'soluble' starch is used as substrate, the optical density D falls approximately exponentially to its final value D_∞, thus simulating a first-order reaction. The apparent velocity constant of the reaction is proportional to the enzyme concentration under the usual conditions of testing, and therefore the activity of the enzyme may be expressed by velocity constant/enzyme concentration. D_∞ is an empirical constant which varies slightly with each preparation of starch, and is only approximately equal to the optical density of the products of reaction.

Q-enzyme activity was determined at 20°, using a test solution prepared from constituents in the proportion: 2 vol. enzyme solution, 1 vol. starch solution (10 mg. 'Analar' soluble starch/ml. of water), 2 vol. citrate buffer solution (0·2 M-disodium hydrogen citrate, NaOH such that the pH of the test solution was 7·0). 0·5 ml. samples were removed at intervals and stained with I_2 (1 mg.)–KI (10 mg.) in a solution of final volume 50 ml. The optical density of the samples was measured in 4 cm. cells with a Hilger-Spekker Absorptiometer (6800 A., Ilford Spectrum Filter no. 608). Log $(D - D_\infty)$ was plotted against time, t (min.), and activities calculated from the slope of the initial straight part of the line using the relation

$$\text{Activity} = -\frac{2{\cdot}30\ \mathrm{d}\log(D - D_\infty)}{c\ \mathrm{d}t},$$

where c is the concentration of the enzyme in the test-solution in mg./ml. The units of activity are therefore min.$^{-1}$ mg.$^{-1}$ ml. If the concentration of the enzyme was not known or was immaterial, the activity of the solution was expressed as $-2{\cdot}30\ \mathrm{d}\log(D - D_\infty)/\mathrm{d}t$ min.$^{-1}$.

Velocities of reaction at 10, 20 and 30° were compared using samples of solution of the crystallized enzyme prepared in 0·2 M-citrate buffer pH 7·0. The tests were carried out simultaneously and curves *A*, *B* and *C* in Figs. 3 and 4 obtained at these respective temperatures. Meanwhile, since *Q*-enzyme is thermolabile (Barker, Bourne & Peat, 1949), a measure of the rate of decay of the enzyme at 30° was obtained by keeping two samples of the enzyme solution at 30° for 88 and 176 min. respectively before adding starch. The velocities of reaction were then measured at 30° to give curves *D* and *E* in Figs. 3 and 4 (p. 184). Control solutions were kept for similar times at 10°.

Reducing power was determined by the method described by Barker *et al.* (1949). The values listed in Table 1 may be compared with one another since, under the standard conditions of testing, the volume of the test solution in each case corresponded to 1·12 times the volume of the potato juice from which the added enzyme was derived.

Phosphorylase was determined under approximately the conditions found by Green & Stumpf (1942) to lead to proportionality between enzyme concentration and phosphate liberation. A solution consisting of 'Analar' soluble starch (0·5 ml. 5% solution), 0·5 M-citrate-NaOH buffer, pH 6·0 (0·5 ml.) and enzyme solution (1·5 ml.) was brought to 35·5°, and 0·1 M-dipotassium glucose-1-phosphate (1 ml.) then added. After 12 min. 6% trichloroacetic acid (5 ml.) was added to the test solution, the precipitated protein centrifuged down, and the supernatant analysed for inorganic phosphate by the method of Allen (1940). Inorganic phosphate not due to the action of the enzyme was allowed for by running a control solution at the same time in which the enzyme had been inactivated by trichloroacetic acid before the addition of glucose-1-phosphate.

RESULTS

Electrophoretic analysis of Q-enzyme prepared by precipitation with lead acetate and ammonium sulphate. A sample of enzyme prepared by the method described by Barker *et al.* (1949) (their fraction 'Q_1') was dialysed for 48 hr. at 0° against 0·01 M-ammonium sulphate, which had been brought to an ionic strength of 0·1 with sodium chloride, and to a pH of 8·2 with ammonia. Analysis in a large sectional cell by the electrophoretic method of Tiselius showed that the constituent proteins were negatively charged and that at least six components were present (Fig. 1*a*). By testing small samples of solution removed from the ascending and descending boundaries, it was further shown that *Q*-enzyme activity was associated only with the protein moving with the highest mobility. This was a very small

fraction of the whole, and from a number of such experiments it was concluded that at the most only 5% of the protein in the preparation was *Q*-enzyme. The very low mobility of the bulk of the protein impurity suggested that a considerable purification could be effected by electrophoresis. This was attempted, but was not pursued when it was found that the enzyme was too unstable in solution to survive the long procedure required.

Using the above figure for the activity of the crystallized enzyme (V), the concentration of *Q*-enzyme in the original potato juice was calculated to be approximately 16 mg./l.

Examination of precipitate III. Before subjecting precipitate III from the ethanol-fractionation process to further fractionation in ammonium sulphate solution, it was examined by electrophoresis, and by the solubility test of Falconer & Taylor (1946). Philpot schlieren photographs (Fig. 1*b*), taken after electrophoresis in 0·1 M-citrate-NaOH solution, pH 7·5, revealed a component of very low mobility representing about 60% of the protein of precipitate III, a component of intermediate mobility (25%), and a relatively fast component (15%). *Q*-enzyme activity was shown to be associated only with the fast component. Precipitate III did not lose any appreciable amount of activity during either electrophoresis or the preliminary dialysis at 0° for 24 hr.

The results of the solubility-activity test carried out on precipitate III at 0° and pH 6·2 in ammo-

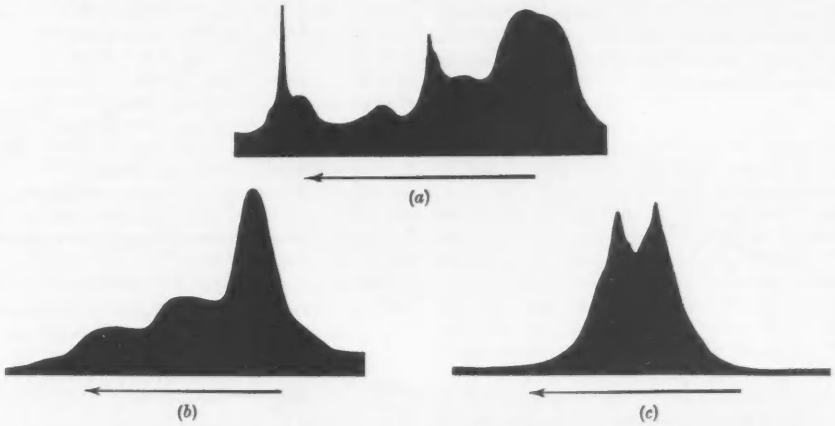

Fig. 1. (*a*) Electrophoresis of *Q*-enzyme prepared by precipitation with lead acetate and ammonium sulphate. Ascending boundary after 124 min. (*b*) Electrophoresis of precipitate III. Ascending boundary after 170 min. (*c*) Electrophoresis of precipitate IV. Ascending boundary after 76 min.

Ethanol and ammonium sulphate precipitation and crystallization of Q-enzyme. The enzyme activity/mg. protein/ml., and the yield of enzyme at different stages of the preparation of crystalline *Q*-enzyme are given in Table 1. Precipitates are numbered as under Methods. The yield is shown relative to precipitate I, since a direct determination of the *Q*-enzyme content of untreated juice is not possible because of the presence of starch precipitants and amylases. The figures are averages of the results of several experiments, each carried out with 100 ml. of potato juice. The relative yield has usually been less if considerably more juice were taken.

Table 1. *Activity and yield at stages in the preparation of crystalline Q-enzyme*

Ppt.	Activity at 20° min.$^{-1}$ mg.$^{-1}$ ml.	Relative yield	Reducing power of test solution after 20 hr. as apparent conversion to maltose (%)	Phosphorylase activity relative to potato juice (=1·0)
I	0·04	1·0	12·5	0·05
III	0·15	0·9	8·6	0·012
IV	0·40	0·7	4·7	<0·007
V	2·4	0·7	1·9	<0·0016

nium sulphate solutions of increasing ionic strength are shown in Fig. 2. Protein began to precipitate at an ionic strength of 3·0 from a solution of 0·18 mg. N/ml. (Kjeldahl). Simultaneously, the activity of the residual solution began to fall, indicating that *Q*-enzyme was present in the least soluble component. According to Fig. 2, section *AB*, the protein precipitated had a constant activity/unit weight until about 80 % of the activity had been precipitated (ionic strength 3·6). A short extrapolation of *AB* to zero activity of the solution showed that if the precipitated component could be treated as homogeneous the enzyme activity was associated with a component representing 34 % of the total protein.

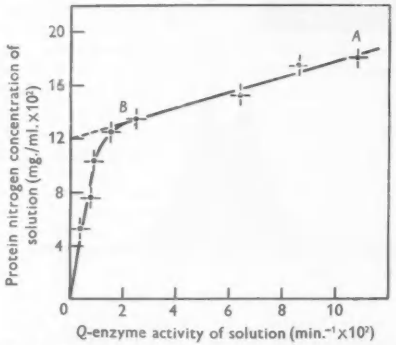

Fig. 2. Solubility-activity curve for precipitate III.

However, in spite of the linearity of *AB*, this component evidently was not a pure protein since the electrophoretic data of Fig. 1*b* had led to a figure of 15 % as a maximum. In conformity with this, electrophoretic analysis (pH 7·5, 0·1 M-citrate-NaOH solution) of the protein precipitated at ionic strength 3·6 (Fig. 1*c*) showed the presence of two components which corresponded in mobility to the two faster components of Fig. 1*b*. There was no sign in Fig. 1*c* of the slow component in Fig. 1*b*, which therefore must remain in solution at ionic strength 3·6.

Stability of Q-enzyme. The crystallized enzyme does not lose more than 5 % of its activity during freeze-drying from 0·05 M-citrate-NaOH buffer pH 7·0 (cf. Barker, Bourne, Wilkinson & Peat, 1950) and is then perfectly stable at 0° in the dry state. In solution in 0·2 M-citrate-NaOH buffer pH 7·0 the activity decays to half in 11 days at 0° or in 4 days at 17°. It will be seen in the next paragraph that the decay at 30° is extremely rapid.

Effect of temperature on the action of Q-enzyme on starch. The relative rates of reaction at 10, 20 and 30° were calculated from the initial slopes of the lines *A*, *B* and *C* respectively in Fig. 4 to be 0·47, 1·0, 1·0. At 30° the activity rapidly decayed during the course of the test, as shown by the progressive decrease in slope of curve *C*, and after 120 min. had fallen to 40 % of its initial value. The two samples of enzyme that had been kept at 30° in the absence of

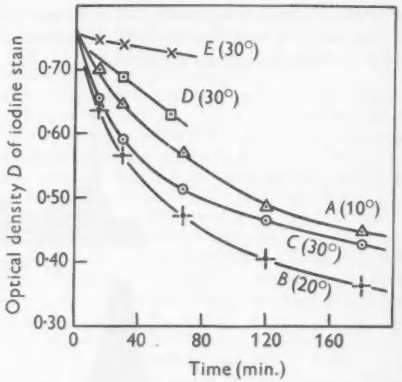

Fig. 3. The effect of temperature on the action of crystallized *Q*-enzyme on starch solutions.

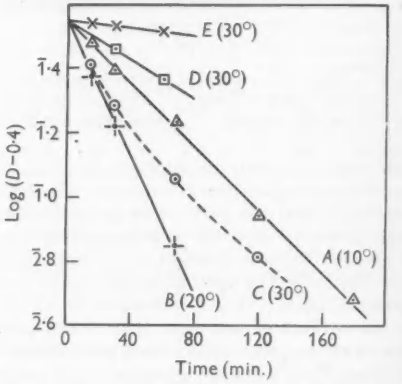

Fig. 4. Activity of *Q*-enzyme. Log $(D - D_\infty)$ as a function of time.

starch for 88 and 176 min., respectively, before being tested, had decayed even more rapidly (curves *D* and *E* respectively) and retained only 29 and 6 % of their activity. The solutions kept at 10° for similar times before the addition of starch showed no loss of activity.

Fresh starch was added to the test solution corresponding to curve *C* after it had stood for 3 hr. at 30° and had cooled slowly to room temperature for 16 hr. Appreciable activity was still present, since the optical density of the iodine stain fell from 0·84 to 0·61 in 120 min. at 20°, and to 0·42 in 270 min.

Activation of Q-enzyme by ions. A suspension of crystals of the enzyme in ammonium sulphate solution was dissolved in water. The ionic strength of the resulting solution was estimated to be 0·017 by passing a sample through an exchange resin and estimating the hydrogen ion produced. The solution was diluted to an ionic strength of 0·010, and then, as a precaution against suspended enzyme which might later be dissolved at higher salt concentrations, filtered through a Whatman no. 42 paper and a G-4 sintered-glass funnel under slight positive pressure. Samples were then added to equal volumes of solutions of starch at pH 7·0 and various ionic strengths equal to or greater than 0·01 (precipitation of the enzyme by reduction in salt concentration thereby being guarded against), and the corresponding activities determined. These are given in Table 2. Further, to test that the effects were not due to suspended enzyme, a portion of the enzyme solution of ionic strength 0·01 was diluted with an equal volume of ammonium sulphate solution of ionic strength 0·01 and the resulting solution tested separately for activation, the results being indicated by the starred entries in the table.

Table 2. *Activation of Q-enzyme by ions. Activity measured in solutions of increasing ionic strength*

Ionic strength of solution	Activity $\times 10^2$ (min.$^{-1}$)
0·01	1·7
0·05	2·9
0·25	4·3
1·25	6·5
0·01*	1·0
1·25*	3·0

* Enzyme solution diluted with $(NH_4)_2SO_4$ solution before testing. See text.

Dependence of the yield of enzyme on the season and on the variety of potato. Difficulty in the preparation of Q-enzyme during a certain period of the year was noted by Barker *et al.* (1949). The same trouble was also encountered in the present investigation. In the middle of March, King Edward potatoes harvested in the previous autumn were found to be almost devoid of active enzyme, although 3 weeks previously their activity had been normal. This change coincided with the beginning of sprouting in the potatoes, and at the same time activity due to an amylase of lower solubility than Q-enzyme disappeared. The amylose precipitant also increased considerably and the precipitates became oily and more difficult to work with. A standard method of fractionation could not be devised until fully grown potatoes were available in August.

With the exception of 'Ninetyfold' potatoes, all the varieties of white-skinned potatoes tested gave very low yields of Q-enzyme in comparison with red-skinned varieties, among which there was little to choose.

DISCUSSION

The original method for preparing Q-enzyme (Bourne & Peat, 1945), after modification by Barker *et al.* (1949), consists of precipitating potato juice with lead acetate, dissolving the precipitated protein and precipitating the enzyme with ammonium sulphate. Although the method has served for the characterization of the enzyme, it is evident from the electrophoresis results above that it does not differentiate sufficiently between the enzyme and the numerous proteins which occur in potato juice. Much greater selectivity is attained by precipitation at low ionic strength with ethanol (Cohn *et al.* 1946, 1950). The number of variables available in this method make it very suitable for problems such as the present one where a minor constituent of a mixture of proteins has to be isolated.

It has to be remembered, however, that ethanol quickly inactivates Q-enzyme if certain limits of temperature and concentration are exceeded (compare the inactivation by ethanol reported by Bourne *et al.* 1945). Even under the conditions described above for the preparation, the enzyme should not be left too long in contact with ethanol, e.g. overnight. Ammonium sulphate solution is therefore a better medium than ethanol solution from which to attempt crystallization.

The influence of the season and of the variety of potato on enzyme content makes it advisable to carry out preliminary experiments before attempting a large-scale extraction of a new batch of potatoes. It is then possible to decide whether sufficient enzyme is present to be worth extraction, and whether any slight alterations in the concentrations of ethanol specified above need be made to achieve optimum results with the particular batch.

The enzymic properties of the crystallized enzyme resemble those described for the amorphous form in the references cited. The ability of the enzyme to form reducing groups when incubated with amylose is somewhat decreased but still remains, although detectable by reducing power measurements only after long incubation. It is confirmed that Q-enzyme is activated by ammonium sulphate as described by Gilbert & Swallow (1949). The experiments were conducted with particular care to eliminate any possibility that enzyme suspended, but not dissolved, at low ionic strengths was passing into solution at higher ionic strengths and simulating activation.

The lability of the enzyme is demonstrated by the results obtained for the reaction with starch at 30°

(curve *C*, Fig. 4). It is obvious that for valid comparisons of reaction rate at different temperatures the initial slope of the reaction curve must be obtained. When this is done it is still found that the velocity of reaction reaches a maximum somewhere between 20 and 30° and that an 'optimum' temperature does not result from the onset of inactivation.

The experiments in which *Q*-enzyme was kept at 30° for some time before adding starch and testing activity confirm this lability. Thus in 2 hr. only 6% of the original activity remained. It is noteworthy that the decay of activity at 30° in the solution which contained starch throughout was much less rapid, a result which suggests that starch or its reaction product tends to stabilize the enzyme.

SUMMARY

1. Potato *Q*-enzyme has been prepared in crystalline form and with high activity by a method involving precipitation with ethanol at low temperature and low ionic strength.

2. Electrophoretic analysis has been used to follow the course of the purification.

3. Activation of the enzyme by ions has been confirmed, and the effect of temperature on the activity of the enzyme has been examined.

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Enzymes of the Potato Concerned in the Synthesis of Starch

2. THE SEPARATION OF PHOSPHORYLASE

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Several accounts have been published of attempts to purify the starch phosphorylase of the potato. The original technique of Hanes (1940), in which the enzyme was precipitated from potato juice with ammonium sulphate, has been modified by, among others, Green & Stumpf (1942), Meyer & de Traz (1944), Weibull & Tiselius (1945), Bourne & Peat (1945) and Barker, Bourne, Wilkinson & Peat (1950).

The degree of purity which can be reached by these methods is probably about 5%, with respect to protein, according to an electrophoretic analysis carried out by Weibull & Tiselius (1945). The electrophoretic analysis would not, however, have differentiated proteins with the same mobility as the enzyme, but a similar estimate from ultracentrifugation experiments by these authors lent support to the figure. The protein impurity is probably mostly inert, but Barker *et al.* (1950) have shown that their preparation still retains considerable phosphatase activity, and that traces of *Q*-enzyme and amylases may be present. A threefold greater purification is reported by Sumner, Chou & Bever (1950) for the phosphorylase of jack bean after a treatment including precipitation from concentrated acetone solution, but only a low yield is claimed.

As described in Part 1 of this series (Gilbert & Patrick, 1952), *Q*-enzyme can be isolated from potato juice in good yield, and in satisfactory purity, by a method of protein purification developed by Cohn *et al.* (1946) and Cohn *et al.* (1950), based on fractionation by ethanol precipitation. Green & Stumpf (1942) and Meyer & de Traz (1944) concluded that ethanol causes complete deactivation of potato phosphorylase even at 0°, but in contradiction to this conclusion it was found that, under the conditions adopted for the preparation of *Q*-enzyme, phosphorylase was stable in the presence of considerable concentrations of ethanol, and could be prepared from the ethanol solution remaining after the precipitation of *Q*-enzyme from potato juice. This residual solution was therefore used as a starting material for the following experiments.

METHODS

Potato juice (100 ml.) was precipitated successively at 11 and 14·9% ethanol concentration as described for the preparation of *Q*-enzyme in Part 1. The supernatant was then adjusted at −5° to a concentration of ethanol of 17·7% by adding 0·087 ml. of 50% (v/v) ethanol-citrate solution (0·01 M-disodium hydrogen citrate, NaOH to pH 6·0)/ml. of supernatant, with the precautions of slow addition and thorough but gentle stirring previously described. After removal by centrifugation at −5°, the precipitate was dissolved at 0° in 20 ml. of 0·01 M-citrate solution of pH 6·0 and then reprecipitated at −5° and 20% ethanol concentration by adding 0·67 ml. of the 50% ethanol-citrate precipitant/ml. The precipitate *A* was dissolved at 0° in 5 ml. of 0·05 M-citrate solution of pH 6·0 and freeze-dried.

The powder (about 100 mg.) was extracted, with gentle but frequent stirring, for 30 min. at −5° with 25 ml. of 20% ethanol-citrate solution (pH 6·0, 0·01 M). After centrifugation at −5°, the insoluble residue was extracted at −2° with stirring for 30 min. with 50 ml. of 11% ethanol solution containing 0·01 M-sodium acetate (HOAc to pH 6·0) (this solution representing a considerable decrease in ionic strength in comparison with the previously used citrate solution of the same molarity). After centrifugation at −2°, the residue, *B*, was extracted for 30 min. with stirring at 0° with 50 ml. of freshly prepared sodium acetate solution (0·01 M, pH 6·0) containing 0·000167 M-zinc acetate. The extracted residue, *C*, was dissolved in 50 ml. of 0·01 M-citrate solution of pH 6·0 and precipitated at 0° from 22% (w/v) $(NH_4)_2SO_4$ by the addition of 0·79 ml. of 50% (w/v) $(NH_4)_2SO_4$ (pH to 6·0 with NH_3) to each ml. of solution. The precipitate was discarded and the supernatant brought to a concentration of 35% (w/v) $(NH_4)_2SO_4$ by the addition of 0·87 ml. of 50% solution/ml. to give precipitate *D*. This precipitate was dissolved in 5 ml. of 0·05 M-citrate buffer solution of pH 6·0 and freeze-dried.

Modification. A higher recovery of the phosphorylase of the potato juice was obtained when the concentration of ethanol for the initial precipitation of the phosphorylase was raised from 17·7% ethanol to 21%, and the precipitate then washed with 30 ml. of 21% ethanol solution containing 0·01 M-citrate of pH 6·0. The effect on the purity of the final product has not yet been studied.

Measurement of enzyme activity. (1) Phosphorylase activity was measured by the method of Green & Stumpf (1942), after slight modification of their conditions, as described in Part 1. The unit used is the weight in mg. of elementary phosphorus produced/min./ml. of test solution for a concentration of protein in the test solution of 1 mg./ml.

(2) A portion of the same solution of precipitate *D* (in 0·01 M-citrate solution, pH 6·0) that was tested for its phosphorylase activity, was tested for *Q*-enzyme and amylase activity by the method of Barker, Bourne & Peat (1949). The amylose test solution (14 ml.) contained 0·05 mg. protein. Protein N in the solution of *D* was determined by precipitation of the protein with trichloroacetic acid, followed by washing to remove $(NH_4)_2SO_4$ and Kjeldahl estimation according to Falconer & Taylor (1946). A factor of 6·25 was used to convert weight of nitrogen to weight of protein. Samples of the digest were examined after 2 and 20 hr. Control solutions were incubated (*a*) with water instead of enzyme solution, (*b*) with water instead of amylose solution.

(3) Phosphatase activity was estimated using, (*a*) sodium glycerophosphate or (*b*) glucose-1-phosphate as substrate.

(*a*) Enzyme solution (5 ml.) was added to 10 ml. of a buffer solution containing 0·15 M-sodium glycerophosphate and 0·2 M-disodium hydrogen citrate (pH to 6·5 with NaOH), and the solution incubated at 20°. A control solution contained water instead of enzyme solution. Free phosphate in these solutions was estimated periodically by the method of Allen (1940).

(*b*) Enzyme solution (12 ml.) was added to a solution prepared from 0·1 M-glucose-1-phosphate dissolved in 0·5 M-citrate-NaOH buffer of pH 6·0 (12 ml.) and 0·1% A.R. soluble starch (4 ml.). The test solution was incubated at 20°. At intervals, 5 ml. samples were withdrawn and tested for liberated glucose by the method of Shaffer & Hartmann (1921). A known weight of glucose (about 0·2 mg.) was added to each 5 ml. sample before this test.

RESULTS

The absolute activities of the residues and precipitates, lettered as under Methods, obtained during the course of the fractionation are given in Table 1.

Table 1. *Activities of phosphorylase preparations*

Preparation	Activity (mg. P/min./ml./ mg. protein/ml.)	Yield relative to ppt. *A*
A	0·15	1
B	0·29	0·9
C	0·61	0·7
D	0·98	0·6

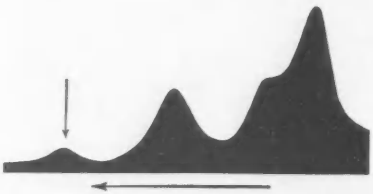

Fig. 1. Electrophoresis of potato phosphorylase after precipitation from potato juice with ethanol (precipitate *A*). An arrow indicates the centre of the phosphorylase boundary.

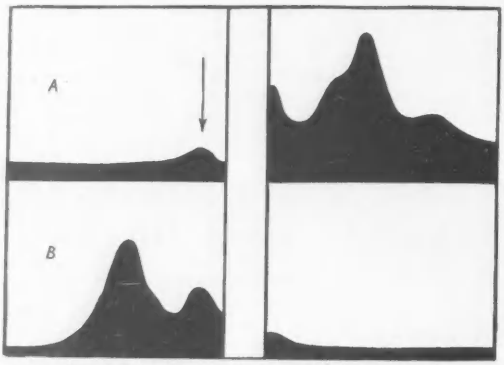

Fig. 2. Philpot-schlieren photograph of sectional electrophoresis U-tube, illustrating the separation of the component (arrow) of precipitate *A* with phosphorylase activity (ascending boundary *A*), and of the components without activity (descending boundary *B*).

Preparation *A* contains about 70 % of the phosphorylase originally in the potato juice, and about 20–30 % of the original *Q*-enzyme. It can be seen that precipitate *D* is about seven times more active with respect to unit weight of protein than preparation *A*.

In the test for amylases carried out on precipitate *D*, no increase in reducing power of the amylose test solution was detected even after incubation for 20 hr. The optical density (6800 A.) of the amylose solution fell from 1·08 to 1·04 in 20 hr. and in the control solution from 1·05 to 1·02. There was thus no indication in these tests of the presence of either amylases or *Q*-enzyme in the phosphorylase preparation.

Electrophoresis of precipitate A. Electrophoretic analysis of precipitate *A* was carried out in 0·1 M-sodium citrate-NaOH buffer at pH 7·0 after dialysis at 0° for 18 hr. The protein migrated towards the anode and was resolved into at least four components during 138 min. (Fig. 1). The fastest of these components, which has been indicated by an arrow in the figure, represented about 6 % of the total protein, the slowest, which hardly moved, about 60 %. By adjusting the positions of the boundaries and sliding the sections of the Tiselius cell, a portion of the fast component was isolated in an electrophoretically pure state from the ascending boundary (Fig. 2). When tested for phosphorylase activity this isolated fraction was found to liberate 0·11 mg. phosphorus/ml. of test solution in 12 min. compared with 0·0024 mg. liberated by the corresponding fraction isolated from the descending boundary which had been freed only from this component. The fast component therefore contained the whole of the phosphorylase.

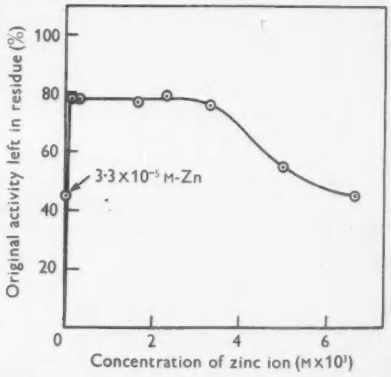

Fig. 3. The residual phosphorylase activity of residue *B* after extraction with dilute solutions of zinc acetate at pH 6·0.

The effect of extraction with zinc acetate solution on the phosphorylase activity of residue B. In the course of determining whether zinc salts could be used to effect any purification of phosphorylase preparations, residue *B* was extracted with 0·01 M-sodium acetate solution, pH 6·0 (50 ml.), containing various quantities of zinc acetate. Determination was then made of the protein content and the activity of the extracted residues and of the solutions. It was discovered that if the concentration of zinc ion was above about $3{\cdot}3 \times 10^{-3}$ M a considerable loss of activity occurred from the residue, but that between this concentration and $1{\cdot}7 \times 10^{-4}$ M-zinc a constant decrease of about 20 % took place as illustrated in

Fig. 3. After the extraction procedure, only the residues were active and no activity was detected in any of the zinc solutions.

In order to throw light on these results, the activity of residue *B* from the stage before the treatment with zinc was measured in a normal test solution and also in a test solution containing 0·02 M-zinc acetate. The rate of liberation of phosphorus proved to be the same, showing that the 20 % loss in activity was not due to any traces of zinc retained in the precipitate during the treatment. (The citrate in the test solution prevented precipitation of the protein by the zinc.)

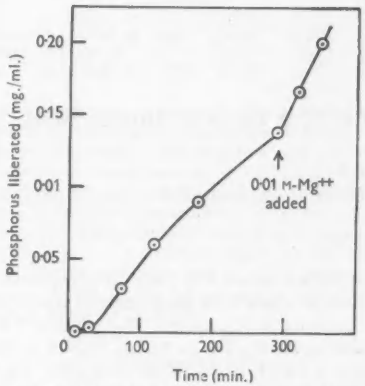

Fig. 4. The hydrolysis of sodium glycerophosphate by partially purified potato phosphorylase (precipitate *A*).

In spite of the loss in total activity incurred, it was decided to include the zinc treatment in the fractionation scheme since there was a net increase in activity/mg. protein (Table 1).

Tests for phosphatase. No phosphatase activity was detected in any of the precipitates or residues when these were incubated with glucose-1-phosphate, and only a normal synthesis of amylose occurred, without a measurable release of reducing sugar. However, when incubated with glycerophosphate, precipitate *A* was found to have considerable phosphatase activity. A solution of the precipitate, concentrated 2·5 times with respect to the potato juice from which it was derived, caused a 4·4 % hydrolysis of 0·1 M-sodium glycerophosphate to free phosphate in 290 min. This rate of hydrolysis was doubled by the addition of magnesium sulphate to 0·01 M. An induction period occurred at the beginning of the reaction (Fig. 4).

Extraction with 11 % ethanol (to give residue *B*) removed almost all the phosphatase, only 0·2 % hydrolysis of 0·1 M-sodium glycerophosphate then occurring in 240 min. The last trace of phosphatase activity was removed (none being detected even with 0·01 M-Mg^{++} in the test solution) during extraction with 0·000167 M-zinc acetate solution in the preparation of residue *C*.

Freeze-drying of precipitate D. At least 92 % of the phosphorylase activity of this precipitate was retained during freeze-drying from solution in 0·05 M-citrate buffer solution of pH 6·0. These are the conditions recommended by Barker *et al.* (1950) except for the use of a lower salt concentration.

DISCUSSION

Fractional precipitation and extraction of potato juice with ethanol, zinc acetate and ammonium sulphate has led to the preparation in reasonable yield of very active phosphorylase. This would seem to have about nine times the activity per unit weight of the preparations discussed by Weibull & Tiselius (1945). Even so, the electrophoretic analysis of precipitate *A*, and the electrophoretic and ultracentrifugation experiments of Weibull & Tiselius on their preparation, suggest that an increase in purity of at least twice remains to be achieved.

The decrease of one-fifth in the activity of phosphorylase during extraction with very dilute solutions of zinc acetate is not yet understood. It could arise from the presence of two rather similar enzymes, only one of which is inactivated by zinc, or from the displacement of an essential cation by zinc, but it cannot be due simply to the retention of traces of zinc by the extracted residue.

Amylase activity was not detected in the purified phosphorylase. The test described, however, cannot be regarded as a satisfactory criterion of purity for phosphorylase required for studies involving amylose of high molecular weight, and it becomes desirable to use measurements of molecular weight directly to prove complete freedom from amylase.

SUMMARY

1. Potato phosphorylase has been prepared by a method involving precipitation from potato juice with ethanol followed by extraction with zinc acetate and precipitation with ammonium sulphate.

2. The phosphorylase preparation has an activity about nine times greater than that of preparations previously described.

3. No phosphatase, amylase or *Q*-enzyme activity has been detected in the product.

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The Component Acids of the Milk Fat of a Grey Atlantic Seal

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The milk fats of many land animals are characterized by the presence of fatty acids of lower molecular weight than those obtaining in their respective depot fats. This phenomenon is most marked in the case of the milk fats of the ruminants which contain appreciable amounts, especially when expressed as a molar percentage, of the lower even-membered saturated acids from butyric to myristic, which are either absent or occur only in small amounts in their respective depot fats. It is not so pronounced, however, in the case of the milk fats of other herbivora, e.g. horse and ass, while sow milk fat has been shown (De la Mare & Shorland, 1944) to resemble closely that of a normal pig depot fat. The Reichert and Polenske values recorded for milk fats of a number of other animals (e.g. cat, dog and mouse) also indicate the absence therefrom of short-chain acids.

Little, however, is known of the composition of the milk fats of marine animals. Klem (1935) has recorded data for various species of whales, and indicated that the fat content of the milk ranged from 19 to 46 %, thus far exceeding that found in the milk of land animals (usually 3–6 %). He also noted that the mean unsaturation of the milk fat of a blue whale was considerably higher (iodine value 171·8) than that of the fat from any other site in the same animal (iodine values 94·0–102·8), though these latter values are considerably lower than the values obtained for fat obtained from the same sites in healthy males and non-lactating females of the same species (iodine values 120–140). He further showed that the blue whale milk fat contained the following acids: myristic 8·4, palmitic 16·8, stearic 1·8, unsaturated acids C_{14} 1·2 (−2·0H), C_{16} 6·2 (−2·0H), C_{18} 26·8 (−3·3H), C_{20} 25·9 (−8·5H), C_{22} 12·9 (−11·0H) % (w/w). Similarly, Schmidt-Nielsen & Frog (1933) showed that there are no acids of lower molecular weight than lauric acid present in the milk fat of the finner whale.

An opportunity for further study of the milk fat of a marine animal was made possible by the kindness of Prof. E. C. Amoroso, Dr S. J. Folley, F.R.S., and Dr S. J. Rowland, who placed a small quantity of the milk of a grey (Atlantic) seal (*Halichoerus grypus*), caught off the Pembrokeshire coast in 1950, at the disposal of this laboratory.

METHODS AND RESULTS

The milk was pale cream in colour, of a thick viscous consistency and had a pronounced fishy odour. On extraction of 235 g. milk with light petroleum (b.p. 40–60°) (emulsions readily broken by addition of ethanol) 118 g. (50·0 %, w/w) of a pale oil, saponification equivalent 295·9, iodine value 146·2, unsaponifiable matter 1·5 %, was obtained. The fat (101 g.) gave 95·2 g. mixed fatty acids, after saponification and acidification of the soaps. The steam distillate from the mixed fatty acids required 1·8 ml. 0·1 N-KOH to neutralize the steam volatile acids, this being equivalent to 0·03 g. decanoic acid (or a proportionally smaller amount of the lower steam-volatile acids) indicating the virtual absence of this group of fatty acids.

Component fatty acids of the seal milk fat

The mixed acids (86·3 g.) were crystallized first from acetone at −70°, the deposited acids being subjected to a further recrystallization from acetone at this temperature. The deposited acids were then crystallized from ether at −40°. In this manner the acids were resolved into three fractions consisting of mainly saturated, mainly monoethenoid and mainly polyethenoid acids respectively, their proportions and unsaturation being recorded in Table 1.

Table 1. *Low temperature crystallization of seal milk fatty acids*

	Weight (g.)	Weight % of total	Iodine value
A Insoluble in ether at −40°	16·85	19·5	3·4
B Soluble in ether at −40°	22·30	25·8	87·5
C Soluble in acetone at −70°	47·15	54·7	234·2

After methylation, according to the procedure recommended by Bjarnason & Meara (1944), each group of esters was fractionally distilled, the analytical data obtained for each ester fraction being recorded in Table 2, together with the spectroscopic analysis of the acids recovered from fractions *B*4 and *C*3. From these results the composition of each ester fraction is calculated according to the methods adequately described by Hilditch (1947, pp. 498–510) and Gupta & Hilditch (1951), and therefrom the composition of each group of acids, leading subsequently to the component acids of the whole fat (Table 3).

DISCUSSION

It is now established that whilst the component fatty acids of whale oils vary but little from season to season (Armstrong & Allan, 1924; Tveraaen, 1935; Hilditch & Maddison, 1948), seal blubber oils show an abnormally large variation in the proportions of their component fatty acids (Hilditch & Pathak, 1949; Winter & Nunn, 1950*a*, *b*, *c*). These latter authors have shown (1950*b*) that significant differences occur, not only in the blubber oils of the elephant seals from different localities (Heard Island and Macquarie Island), but also in the blubber oils of elephant seals caught in the same locality (Heard Island) and concluded that the influence of any of the three factors, diet, sex and species, was not sufficient to account for the observed differences.

Comparison of the component acids recorded in Table 4 shows that no fundamental difference exists between the blubber oils of the seals so far reported and the seal milk fat, such as occurs between the depot and milk fats respectively of the ruminants and some other land animal species.

There are, however, notable differences in the proportions of the component acids of the seal milk fat and the blubber fat of an animal of the same species, and indeed differences from the blubber fats of all the species of seals so far recorded. Thus, although the content of both myristic and stearic acids in the milk fat is of the same order as that which obtains in the blubber oils of both British coastal and Antarctic seals, the milk fat differs markedly, in that the palmitic acid content rises to about 16% as compared with the more usual 10–12% in the blubber oils. This increase in the palmitic, and therefore in the total saturated acid content of the milk fat does not appear to be at the expense of any particular unsaturated acid group, if the recorded analysis of grey seal blubber fat can be considered to be typical for that species.

Table 2. *Fractionation data for seal milk ester fractions*

Fraction	Weight (g.)	Saponification equivalent	Iodine value
	Methyl esters of acids *A*		
*A*1	1·40	245·8	1·1
*A*2	1·87	266·8	0·8
*A*3	2·55	269·1	1·0
*A*4	2·60	269·3	0·8
*A*5	3·00	270·5	0·5
*A*6	3·40	278·0	5·6
*A*7	2·22	333·1*	16·4
Total	17·04	—	
	Methyl esters of acids *B*		
*B*1	1·77	262·0	52·8
*B*2	2·05	286·0	78·2
*B*3	2·06	294·8	82·3
*B*4	2·70	295·3	83·7
*B*5	2·59	295·3	83·9
*B*6	2·80	297·1	85·5
*B*7	3·19	297·3	85·8
*B*8	2·77	298·7	86·1
*B*9	2·72	323·2*	96·9
Total	22·65	—	—
	Methyl esters of acids *C*		
*C*1	1·67	241·6	64·2
*C*2	2·31	263·0	89·4
*C*3	2·80	267·5	98·0
*C*4	3·61	271·0	98·2
*C*5	4·42	281·0	106·7
*C*6	6·51	292·3	111·6
*C*7	7·01	302·2	143·8
*C*8	6·83	325·8	337·6
*C*9	3·57	334·9	389·6
*C*10	3·38	336·9	386·9
*C*11	2·30	338·2	376·4
*C*12	3·47	363·8*	233·3
Total	47·88		

* Equivalents of esters (freed from unsaponifiable matter): *A*7, 300·4; *B*9, 306·7; *C*12, 330·4.

Spectroscopic analysis of acids recovered from fractions *B*4 and *C*3

	*B*4	*C*3
$E^{1\%}_{1\,cm.}$, 232 mμ. (unisomerized)	—	5
$E^{1\%}_{1\,cm.}$, 270 mμ. (unisomerized)	—	1*
$E^{1\%}_{1\,cm.}$, 234 mμ. (180°/60 min.)	3*	42
$E^{1\%}_{1\,cm.}$, 268 mμ. (170°/15 min.)	1*	17

* Neglected.

Table 3. *Component acids in groups A, B, C, and in the whole seal milk fat*

Acid	*A* (19·5%)* %†	*B* (25·8%)* %†	*C* (54·7%)* %†	Total fatty acids excluding unsaponifiable (%, w/w)	(% by mol.)
Myristic	8·7	0·9	1·5	2·8	3·4
Palmitic	71·0	4·5	2·2	16·4	17·8
Stearic	14·4	—	—	2·8	2·8
Unsatd. C_{14}	0·1 (−2·0)	0·8 (−2·0)	2·6 (−2·0)	1·7 (−2·0)	2·0
Unsatd. C_{16}	0·5 (−2·0)	5·3 (−2·0)	20·3 (−2·2)	12·7 (−2·2)	13·9
Unsatd. C_{18}	2·5 (−2·0)	80·3 (−2·0)	27·8 (−2·7)	36·6 (−2·3)	36·3
Unsatd. C_{20}	1·4 (−2·0)	7·6 (−3·7)	20·5 (−7·2)	13·6 (−6·6)	12·4
Unsatd. C_{22}	—	—	24·4 (−11·2)	13·4 (−11·2)	11·4
Unsaponifiable	1·4	0·6	0·7	—	—

* Group as % (w/w) of total fat.
† Component acids as % (w/w) of group.
Figures in brackets denote mean unsaturated of each group of acids (Hilditch, 1947, p. 23).

Table 4. *Component fatty acids of seal blubber oils and grey seal milk fat* (%, *w/w*)

	Elephant seal, Macquarie Island		Elephant seal, Heard Island	Leopard seal, Heard Island	Common seal, Norfolk coast		Grey Atlantic seal, Pembroke coast	
	Mature male	Mature female	mature male	mature male	Suckling female	Yearling male	Blubber fat	Milk fat
Iodine value of oil	136·4	153·5	114·8	130·7	140·4	145·4	162·2	146·2
Acid:								
Lauric	0·3	0·1	—	—	—	—	—	—
Myristic	5·2	4·4	3·4	4·0	2·4	2·2	3·7	2·8
Palmitic	11·4	11·5	9·6	7·4	10·3	10·6	10·5	16·4
Stearic	2·4	2·4	2·5	1·6	2·5	4·4	2·0	2·8
Arachidic	—	—	0·3	0·2	0·3	0·3	—	—
Unsatd. C_{14}	1·7	1·3	1·2	2·1	2·9	2·2	1·6	1·7
Unsatd. C_{16}	14·5	13·0	10·5	12·7	25·7	20·8	15·5	12·7
Unsatd. C_{18}	35·1	33·0	39·8	42·3	32·6	33·7	30·8	36·6
Unsatd. C_{20}	18·8	20·6	18·0	17·3	12·1	13·6	16·5	13·6
Unsatd. C_{22}	10·6	13·7	14·7	12·4	11·2	12·2	18·1	13·4
Unsatd. C_{24}	—	—	—	—	—	—	1·3	—
Mean unsaturation of:								
Unsatd. C_{14}	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0
Unsatd. C_{16}	−2·1	−2·1	−2·1	−2·1	−2·1	−2·2	−2·2	−2·2
Unsatd. C_{18}	−2·7	−2·4	−2·2	−2·2	−2·7	−2·4	−2·7	−2·3
Unsatd. C_{20}	−5·4	−5·9	−4·0	−4·6	−5·9	−7·2	−5·7	−6·6
Unsatd. C_{22}	−10·0	−10·8	−7·4	−9·4	−11·0	−11·0	−10·6	−11·2
Unsatd. C_{24}	—	—	—	—	—	—	−11·0	—

Although there is considerable variation in the amounts of the unsaturated acids of the various blubber oils, the grey seal milk fat does not conform to any type so far examined. Thus the C_{16} unsaturated acids more nearly resemble those of the blubber fats of the Antarctic seals rather than those from British coastal waters, whereas curiously, with the exception of leopard seal blubber, the C_{18} unsaturated acid content of the milk fat shows the least resemblance to that of the grey seal blubber fat. Again, although there is no correlation between the C_{20} unsaturated acids with those of the Antarctic species, the milk fat much more nearly resembles those values recorded for the blubber of the common seal than that of the grey seal, this latter observation also holding true for the C_{20} unsaturated acids.

It appears, therefore, that although they are broadly similar there may be no simple correlation between the milk fat of the grey seal and that of its blubber fat, neither does any correlation exist with the blubber fat of other species so far examined.

A further point of interest lies in the fact that the grey seal milk fat is considerably less unsaturated (iodine value 146·2) than that recorded for grey seal blubber oil (iodine value 162·2). In this respect seals might seem to differ entirely from whales, since the milk fat of a blue whale was found to be more unsaturated than that of the fat from any other site in the body of the same animal.

Clearly much more information is necessary before the apparent abnormalities of seal oils are understood, and to that end it is hoped that in the

near future an opportunity may be afforded of studying the blubber and milk fats of a seal at various stages during lactation, and also the blubber fat of the calf.

SUMMARY

1. Grey (Atlantic) seal (*Halichoerus grypus*) milk resembles whale milk in that its fat content is of the order of ten times that found in the milks of land animals.

2. The composition of seal milk fat has been investigated: it contains only those acids which occur in its blubber fat.

3. Qualitatively grey seal milk fat resembles that of the blubber oil of the same and of a number of other species, notable differences, however, occurring in the proportions of palmitic and unsaturated acids.

4. Grey seal milk fat is relatively more saturated than that of the blubber oil in contradistinction to that of the blue whale, for which the reverse holds true.

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The Liberation of Chloride Ions from Organic Chloro Compounds by Tissue Extracts

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There have been several reports of the liberation of chloride ions from organic chloro compounds. Liebreich (1869), who introduced chloral hydrate as a hypnotic, stated that this compound was in part excreted as inorganic chloride. This was disputed by von Mering & Musculus (1875), von Mering (1882) and Külz (1882) who found that chloral hydrate was eliminated by man and dogs as urochloralic acid (trichloroethyl-β-glucuronide). Kast (1887) failed to find an increased chloride excretion after administration of chloral hydrate to dogs, although he observed that chloride excretion was considerably increased after chloroform anaesthesia. The increase in man was slight. Willstätter, Straub & Hauptmann (1922) detected only small amounts of urochloralic acid in urine when therapeutic doses of chloral hydrate were given to man or rabbits. They maintained that urochloralic acid was formed when large doses were given, but that chloral was broken down mainly to inorganic constituents when therapeutic doses were given. The only metabolic product detected in urine after giving trichlorourethane to man, rabbits or dogs was inorganic chloride. Akamatsu & Wasmuth (1923), using rabbits, showed that even after large doses of chloral hydrate no more than half could be accounted for as urochloralic acid. More recently Heppel & Porterfield (1948) claimed the discovery of an enzyme in rat liver, kidney and spleen which liberated halide ions from halogenated aliphatic hydrocarbons.

Since metabolic studies on a number of chloro-compounds were being undertaken in this laboratory it was desirable to confirm the existence of an enzyme such as that claimed by Heppel & Porterfield, especially as these workers did not appear to have considered the possibility of chloride ions being liberated as a result of interaction between chloro compounds and SH compounds. Several instances

of reaction between halogen compounds and SH compounds according to the general scheme

$$R.SH + R'.Cl \rightarrow R.S.R' + H^+ + Cl^-$$

have been reported, e.g. halogenated lachrymators (Dixon, 1950), halogenated acetic acids (Dickens, 1933; Rapkine, 1933; Quastel, 1933; Michaelis & Schubert, 1934), lewisite and 2:3-dimercaptopropanol (Thompson, 1950), formation of benzyl mercapturic acid from benzyl chloride in the rabbit (Stekol, 1939). The work here reported describes a preliminary investigation of the liberation of chloride ions from 29 chloro compounds, and a more detailed study of the effect of SH compounds upon the liberation of chloride ions from three of those compounds.

METHODS

Materials. The chloro compounds were all purchased (British Drug Houses Ltd.). DL-Cysteine hydrochloride and L-cystine (British Drug Houses Ltd. and Light and Co. Ltd.) were used. Glutathione was obtained from Roche Products Ltd. and The Distillers Co. Ltd. A phosphate buffer, pH 6·8 (equal vol. Na_2HPO_4 and NaH_2PO_4, 0·2 M), was used unless otherwise stated. The phosphoric-tungstic acid solution was prepared by dissolving 6 g. $Na_2WO_4.2H_2O$ in 1 l. 0·15 M-H_3PO_4.

Extracts. The animal was killed by a sharp blow on the back of the neck and immediately bled. The liver was removed, weighed and ground with acid-washed sand (British Drug Houses Ltd.) and water (1·5 × the original weight). The resulting brei was centrifuged and the supernatant liquid decanted and used immediately.

Digests. NaCl (55 mg./100 ml.) was dissolved in phosphate buffer to ensure that the concentration of Cl^- in the final digest was not less than 3 mM. Chloro compound, to give the required final concentration (usually 0·024 M), was added to a mixture of the buffer (15 ml.) and extract (10 ml.). Three drops of toluene were added as preservative. (Control experiments had shown that, up to 7 hr., toluene had no significant effect upon the liberation of Cl^-.) Chloro compounds insoluble in the mixture were dissolved in ethanol (2·5 ml.) and the volume of buffer reduced to 12·5 ml. As will be seen later, ethanol causes slight inhibition of the liberation of Cl^- from chloral hydrate. The digests were incubated at 37·4° in stoppered flasks. Control solutions were: (*a*) the digest with the extract replaced by a solution of NaCl (70 mg./100 ml.), and (*b*) the digest without chloro compound. The first gave a measure of the spontaneous liberation of Cl^-. A control with boiled extract was not used for the reason stated below. The usual concentration of SH compounds was 0·02 M and when these were used, control digests without chloro compound were set up to give some indication of the extent of loss of SH-groups due to atmospheric oxidation, since it was impracticable to carry out the digestions, withdrawal of samples and analyses in an atmosphere of N_2.

Estimation of chloride ions. The method finally adopted was essentially a modification of that described by Van Slyke & Hiller (1946), which was effective with concentrations of Cl^- over 3 mM. Digest (2 ml.) was thoroughly mixed with phosphoric-tungstic solution (5 ml.) and $AgIO_3$ (60 mg.) and centrifuged. The supernatant liquid was separated, filtered and titrated (2 ml. portions with starch as indicator) against 0·01 N-$Na_2S_2O_3$) immediately after addition of 200 mg. NaI. This method gave good results in the absence of tissue extracts, but recovery of added Cl^- tended to be low by up to 5% in the presence of extracts. The complete liberation of one Cl atom from 1 mol. substrate under the above conditions should cause an increase in titre of 8·21 ml. $Na_2S_2O_3$. Calculations have been based upon the assumption that this titre represents 100% liberation of Cl^-. Values significantly greater than 100% thus indicate the liberation of more than one Cl atom/mol. substrate. Under the conditions used, added SH compounds did not interfere, provided that titrations were carried out within 20 min. of the addition of $AgIO_3$. In some experiments the results by this method were confirmed by a polarographic method based upon that of Zimmerman & Layton (1949). For these, trichloroacetic acid solution (5%, w/v) was used in place of the phosphoric-tungstic acid solution.

Estimation of SH *content.* The method was based on those of King, Baumgartner & Page (1930), Kühnau (1931) and Lucas & King (1932). Digest (2–5 ml. according to SH content) was added to trichloroacetic acid (10%, w/v) to give total vol. 20 ml. The protein precipitated was removed by centrifugation (1 min. at 3000 rev./min.) followed by filtration. Filtrate (10 ml.) was cooled to 0° and mixed with 1 ml. KI (10%w/v) and 3 ml. 0·01 N-I_2. Excess I_2 was titrated with 0·002 N-$Na_2S_2O_3$ using starch as indicator. The I_2 consumption was referred to calibration curves from which the SH content was calculated. Calibration curves were prepared from buffered (pH 6·8) NaCl solution of the SH compounds concerned in concentrations up to 0·026 M. Glutathione (0·006 M) was used for the calibration curve when cystine was added to digests. Confirmation of the presence and disappearance of SH groups was obtained by the nitroprusside test (Hopkins, 1921).

Estimation of urease. The method was essentially that of Marshall (1913), using urease tablets (British Drug Houses Ltd.) as the source of urease.

Estimation of papain. The milk-clotting method of Balls & Hoover (1937) was modified by mixing the enzyme with buffer (pH 4·6) and using fresh cow's milk (10 ml.) in place of the dried-milk solution.

RESULTS

Chloral hydrate and trichloroacetamide

Chloral hydrate (2:2:2-trichloro-1:1-dihydroxyethane) and trichloroacetamide were selected as substrates for the initial experiments. The former liberates chloride ions spontaneously, but the latter is stable in solution at pH 6·8 in the absence of liver extract.

Activity of liver extracts of various species. Digests were prepared with extracts of rabbit, guinea pig and rat liver. Owing to the small size, rat-liver extract was made with 2·5 parts water. The digests were incubated for 24 hr., samples being withdrawn at intervals. The results are shown in Table 1, which also shows the extent of the spontaneous liberation of chloride ions from chloral hydrate. Trichloroacetamide digests included ethanol (10%, v/v).

Table 1. *Liberation of chloride ions from chloral hydrate and trichloroacetamide caused by liver extracts*

(Substrates 0·024 M, pH 6·8. Results expressed as the percentage of 1 Cl/mol. Ranges are given in parentheses below the average values.)

Source of liver extract	No. of samples tested	Percentage of chloride liberated 1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
		Chloral hydrate				
Rabbit	19	2·9 (1·0–5·2)	4·7 (2·1–7·3)	7·2 (4·1–9·7)	11·5 (7·2–16·1)	14·9 (9·8–20·7)
Guinea pig	6	2·7 (1·2–4·1)	4·8 (2·3–6·7)	8·0 (4·5–10·1)	13·9 (9·3–16·3)	18·0 (13·0–19·9)
Rat	6	1·1 (0·8–1·4)	1·7 (1·4–2·3)	2·8 (2·3–3·9)	4·9 (3·9–6·9)	6·7 (4·8–9·2)
None	7	0·2 (0·2–0·3)	0·4 (0·3–0·5)	0·8 (0·7–0·9)	1·9 (1·5–2·2)	4·6 (3·5–5·4)
		Trichloroacetamide				
Rabbit	6	1·2 (0·9–1·5)	2·4 (1·7–3·0)	4·4 (2·9–5·5)	9·1 (5·2–11·6)	13·1 (7·3–16·8)

Effect of pH. Digests with chloral hydrate were made up with phosphate buffer of pH varying from 6·2 to 8·0. Liberation of chloride ions in the former was small, but an increased liberation at pH 8·0 was largely due to spontaneous decomposition of the chloral hydrate. Results from a typical experiment are shown in Fig. 1. It is clear that the pH (6·8) selected for routine use is one at which liver extract causes appreciable liberation of chloride ions, whilst the extent of spontaneous decomposition is low.

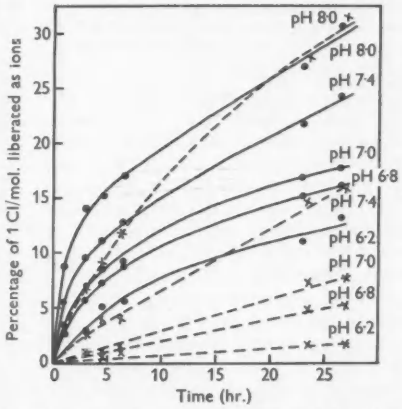

Fig. 1. The effect of pH upon the liberation of chloride ions from chloral hydrate (0·024 M) by rabbit-liver extract (unbroken lines). Spontaneous liberation of chloride ions in absence of extract shown by broken lines.

Effect of boiling. Controls with boiled extracts were not used, since the extracts showed considerable activity even after boiling 1 hr. under reflux. (The coagulum formed on boiling was finely ground before incubation.) The results of a typical experiment with chloral hydrate are shown in Fig. 2 and similar results were obtained with trichloroacetamide in digests including ethanol (10%, v/v). Digests which liberated 8% chloride after 24 hr. with unboiled extract, liberated 6% when the extract had been previously boiled.

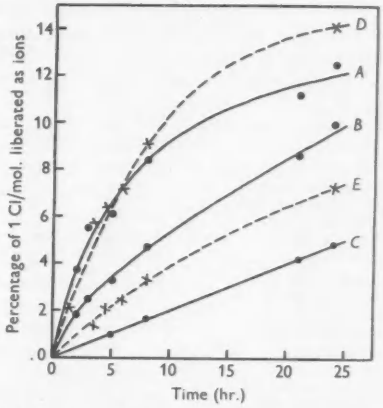

Fig. 2. The effect of boiling (unbroken lines) and of dialysis (broken lines) upon the chloride-liberating activity of rabbit-liver extract. Substrate, chloral hydrate (0·024 M) at pH 6·8. Curve *A*, digest with untreated extract. Curve *B*, digest with the extract previously boiled 1 hr. Curve *C*, spontaneous liberation of chloride ions from chloral hydrate. Curve *D*, digest with another untreated extract. Curve *E*, digest with this extract previously dialysed 20 hr. against running water.

Effect of dialysis. Extracts were dialysed against running water for 20 hr. and their activity compared with that of the original extract which had been kept at the same temperature. In every case some loss of activity was observed, but the dialysed

extract always retained some activity. The results of an experiment with chloral hydrate are shown in Fig. 2. Similar results were obtained with trichloroacetamide, about half of the chloride-liberating activity of the extract being retained after dialysis.

Effect of ethanol. Since many of the chloro compounds used were sparingly soluble in the absence of ethanol, the effect of the addition of ethanol to chloral hydrate digests was examined. It is clear from Table 2 that the inclusion of ethanol (10% of total vol.) caused some inhibition of the

Table 2. *Liberation of chloride ions from chloral hydrate caused by rabbit-liver extracts with and without the addition of ethanol*

(Chloral hydrate 0·024 M, ethanol 10% of total volume, pH 6·8. Results expressed as the percentage of 1 Cl/mol. Ranges are given in parentheses below the average values.)

Digest	No. of samples tested	Percentage of chloride liberated				
		1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Extract + chloral hydrate	7	3·1 (2·1–4·1)	5·1 (3·8–6·1)	8·1 (6·3–9·2)	13·2 (9·9–16·1)	17·3 (13·6–20·7)
Extract + chloral hydrate + ethanol	8	2·6 (1·1–4·2)	4·2 (2·1–6·5)	6·5 (3·7–9·4)	10·2 (6·3–14·1)	13·0 (9·4–17·9)
Chloral hydrate	7	0·2 (0·2–0·3)	0·4 (0·3–0·5)	0·8 (0·7–0·9)	1·9 (1·5–2·2)	4·6 (3·5–5·4)
Chloral hydrate + ethanol	4	0·2 (all 0·2)	0·4 (0·3–0·5)	0·8 (0·7–0·9)	1·9 (1·7–2·1)	4·6 (4·0–5·0)

Table 3. *Liberation of chloride ions from various chloro compounds by rabbit-liver extracts*

(Substrates normally 0·024 M in 10% ethanol, pH 6·8. Results expressed as a percentage of 1 Cl/mol.)

Substrate	Percentage of chloride liberated									
	With extract					Without extract				
	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Chloroform	0·6	1·1	2·0	3·9	6·4	0·0	0·1	0·2	0·4	1·2
Benzyl chloride	9·3	14·8	22·0	35·1	52·8	4·6	8·4	14·3	26·2	46·9
Benzotrichloride	23·5	38·1	62·5	120·2	193·1	28·7	46·9	71·9	108·0	159·8
Ethylene dichloride (1:2-Dichloroethane)*	0·0	0·0	0·1	0·3	0·8			None		
1:1:2:2-Tetrachlorethane	0·7	1·3	2·4	5·4	12·6	0·2	0·4	0·8	2·1	5·2
Pentachloroethane	2·0	3·7	6·7	15·1	31·9	1·1	2·1	3·8	7·1	13·0
Hexachloroethane	2·3	4·1	7·0	13·5	23·3			None		
Trichloroethylene	0·0	0·1	0·2	0·5	1·1			None		
Ethylene chlorohydrin (2-Chloroethanol)†	0·3	0·5	0·8	1·4	2·3	0·0	0·1	0·2	0·5	1·2
Trichloroacetic acid††‡	1·1	1·8	3·0	4·9	6·9			None		
Trichloroacetamide	1·4	2·8	5·3	11·0	16·8			None		
Chloral hydrate†	4·1	5·9	8·1	12·3	15·0	0·2	0·4	0·8	1·9	4·6
Chloralose	1·1	2·2	4·1	7·9	11·3			None		
ω-Chloroacetophenone	4·5	7·5	13·0	25·1	43·2	0·1	0·2	0·4	1·1	2·5
1:2-Dichloropropane	0·2	0·4	0·6	1·3	2·0	0·0	0·1	0·2	0·5	1·1
1-Chloropropionic acid††‡	1·1	1·9	2·9	4·6	6·2	0·1	0·2	0·4	0·9	2·1
2-Chloropropionic acid††‡	2·3	3·7	5·6	9·0	11·6	0·4	0·8	1·6	4·1	9·6
1:3-Dichloroacetone†	40·8	52·7	70·0	101·5	127·8	9·9	19·7	40·0	85·6	130·1
Chloretone	0·9	1·6	2·8	6·2	13·3	0·1	0·2	0·4	0·9	2·3
Butyl chloral hydrate	13·1	25·3	36·1	45·9	49·6	0·2	0·4	0·7	1·9	4·5
iso-Amyl chloride§ (1-Chloro-3-methylbutane)	0·1	0·2	0·2	0·3	0·4			None		
Chlorobenzene	0·5	0·6	0·9	1·4	1·9			None		
p-Dichlorobenzene	0·9	1·4	1·9	2·3	2·8			None		
o-Chlorobenzoic acid††‡	0·4	0·6	0·8	1·3	1·5			None		
m-Chlorobenzoic acid††‡	1·1	1·9	2·5	3·1	3·3			None		
p-Chlorobenzoic acid††‡	0·8	1·3	1·7	2·0	2·1			None		
o-Chlorobenzamide	0·3	0·5	0·9	1·7	2·7			None		
m-Chlorobenzamide	0·1	0·2	0·3	0·8	1·6			None		
p-Chlorobenzamide	0·3	0·5	0·9	1·7	2·8			None		

* 0·036 M.
† Digests did not contain ethanol
‡ Substrate made up with 1 equiv. $NaHCO_3$.
§ 0·072 M.

liberation of chloride ions, although it had no effect upon the spontaneous decomposition of chloral hydrate.

Other chloro compounds

Rabbit-liver extract was used in the examination of twenty-seven other chloro compounds. In every experiment a digest with chloral hydrate was used to confirm the activity of the liver extract. Typical results for each of the twenty-nine compounds used are given in Table 3. It is clear that the extent of liberation of chloride ions by liver extract is not directly related to the ease of spontaneous liberation of chloride ions from a compound. Hexachloroethane, trichloroacetamide and chloralose (1-(2′:2′:2′-trichloro-1′-hydroxyethyl)-1:6-anhydroglucopyranose), yield a considerable percentage of their chlorine as ions with tissue extract but none spontaneously. Some compounds, butylchloral hydrate (2:2:3-trichloro-1:1-dihydroxybutane), ω-chloroacetophenone, chloral hydrate and chloretone (1:1:1-trichloro-2-hydroxy-2-methylpropane), yield small amounts of chloride ions spontaneously but much more in the presence of tissue extract. Other compounds which readily liberate considerable amounts of chloride spontaneously, benzyl chloride, benzotrichloride (ω-trichlorotoluene), and pentachloroethane, liberate still more with tissue extract. 1:3-Dichloroacetone, which liberates more than half its chlorine as ions spontaneously, does not liberate any more after 24 hr. in the presence of liver extract, although the extract accelerates the initial liberation of chloride ions. Several compounds, including those in which chlorine is directly attached to the benzene ring, liberate no significant amount of chloride either spontaneously or with liver extract.

Activity of liver extracts after repeated injection of chloral hydrate

Heppel & Porterfield (1948) observed that the livers of rats which had been repeatedly exposed to an atmosphere containing bromochloromethane caused a greater increase in liberation of bromide ions than the livers of untreated animals. Experiments with this object were carried out with chloral hydrate upon guinea pigs and rats. Six animals were given thirteen injections of chloral hydrate (25 mg.) subcutaneously on alternate days. The animals were then killed and the activity of the livers compared with those of six animals kept as controls. The activity of the livers of the treated animals was slightly less than those of the control animals. In no experiment was increased activity observed. A typical experiment with guinea pigs (350–450 g. body weight) is shown in Table 4.

Table 4. *Effect of previous injection of guinea pigs with chloral hydrate upon the liberation of chloride ions from chloral hydrate caused by extracts of their livers*

(Substrates 0·024 M, pH 6·8. Results expressed as percentage of 1 Cl/mol. Ranges are given in parentheses below the average values. There were six animals in each group.)

Source of liver extract	Percentage of chloride liberated				
	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Injected animal	2·6 (1·4–4·4)	4·2 (2·6–6·1)	6·5 (4·6–8·7)	11·0 (8·5–13·8)	15·3 (11·7–18·6)
Untreated animals	2·7 (1·2–4·1)	4·8 (2·3–6·7)	8·0 (4·5–10·1)	13·9 (9·3–16·3)	18·0 (13·0–19·9)

Experiments with SH *compounds*

When chloral hydrate is incubated with liver extract there is in addition to the liberation of chloride ions, a loss in the SH content of the digest. This can be seen from the results recorded in Table 5 which gives the liberation of chloride ions and the loss of SH in m-equiv./l. digest. While the differences between the chloride values give the increased liberation of chloride ions due to reaction of chloral hydrate with extract, the differences between SH values are unlikely to give a measure of the SH compounds combining with chloral hydrate since the reaction with chloral is obviously very rapid compared with the reactions (e.g. atmospheric oxidation) causing disappearance of SH from extracts in the absence of chloral hydrate. For example 40% of the total SH is lost in the first hour when chloral hydrate is present, but there is no detectable loss in the absence of chloral hydrate. It should also be remembered that the method for SH estimation determines only SH soluble in trichloroacetic acid and that any SH groups bound to protein would be precipitated and removed before titration. The results do, however, indicate that the liberation of chloride ions involves a loss in the SH content of the extract.

The effect of the addition of cysteine. It can be seen from the results of a typical experiment in Fig. 3 that cysteine in the absence of liver extract causes liberation of chloride ions and that if liver extract is also present the chloride liberation is much greater than when either extract or cysteine alone is present. Table 6 summarizes the results of some experiments with chloral hydrate and cysteine with

Table 5. *Average liberation of chloride ions from chloral hydrate and the average loss in* SH *content of rabbit-liver extract when incubated together*

(Chloral hydrate 0·024 M, pH 6·8, 37°. Results expressed in m-equiv./l. digest and ranges given in parentheses.)

Digest	No. of samples tested		1 hr.	2 hr.	4 hr.	10 hr.	Initial SH content
Extract + chloral hydrate	16	Cl^- liberated	0·9 (0·5–1·1)	1·4 (0·9–1·8)	2·1 (1·3–2·5)	3·2 (2·3–3·6)	—
Chloral hydrate alone	4	Cl^- liberated	0·0 —	0·1 (0·1)	0·2 (0·1–0·3)	0·5 (0·4–0·7)	—
		Difference	0·9	1·3	1·9	2·7	—
Extract + chloral hydrate	8	SH lost	0·9 (0·1–1·4)	1·2 (0·2–1·8)	1·5 (0·5–2·2)	1·7 (0·9–2·5)	2·3 (1·3–2·9)
Extract alone	8	SH lost	0·0 (+0·4*–0·3)	0·2 (+0·4*–0·5)	0·6 (0·1–0·9)	1·2 (0·6–1·8)	2·3 (1·3–2·9)

* +indicates a gain in SH content.

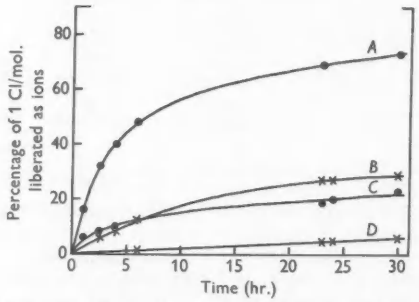

Fig. 3. The effect of cysteine (0·02 M) upon the liberation of chloride ions from chloral hydrate (0·024 M) by rabbit-liver extract at pH 6·8. Curve *A*, digest with chloral hydrate, extract and cysteine. Curve *B*, digest with chloral hydrate and cysteine. Curve *C*, digest with chloral hydrate and extract. Curve *D*, spontaneous liberation of chloride ions from chloral hydrate at pH 6·8.

Table 6. *Average liberation of chloride ions from chloral hydrate and average loss in* SH *content of cysteine when incubated with and without rabbit-liver extract*

(Chloral hydrate 0·024 M, cysteine 0·02 M, pH 6·8 at 37°. Results expressed in m-equiv./l. digest and ranges given in parentheses.)

Digest	No. of samples tested		1 hr.	2 hr.	4 hr.	10 hr.	Initial SH content
Cysteine + chloral hydrate	6	Cl^- liberated	0·6 (0·5–0·8)	1·2 (0·9–1·4)	2·2 (1·7–2·5)	4·7 (3·7–5·0)	—
Chloral hydrate alone	4	Cl^- liberated	0·0	0·1 (0·1)	0·2 (0·1–0·3)	0·5 (0·4–0·7)	—
		Difference	0·6	1·1	2·0	4·2	—
Cysteine + chloral hydrate	4	SH lost	1·1 (0·4–1·6)	1·7 (1·0–2·4)	3·4 (2·5–4·0)	8·6 (8·5–9·1)	20·3 (18·9–22·5)
Cysteine alone	4	SH lost	0·5 (0·2–0·6)	1·0 (0·7–1·2)	2·1 (1·6–2·4)	5·5 (4·9–6·2)	20·2 (18·9–22·5)
		Difference	0·5	0·7	1·3	3·1	—
Extract + cysteine + chloral hydrate	3	Cl^- liberated	3·2 (2·8–3·7)	5·8 (5·0–6·3)	9·2 (7·9–10·0)	13·6 (12·6–14·8)	—
		Difference*	3·2	5·7	9·0	13·1	—
Extract + cysteine + chloral hydrate	3	SH lost	3·9 (2·5–4·6)	6·4 (4·5–7·5)	10·0 (7·6–11·8)	15·2 (13·7–16·7)	20·5 (19·6–21·0)
Extract + cysteine	3	SH lost	1·0 (0·7–1·4)	1·9 (1·4–2·7)	3·6 (2·9–4·6)	8·3 (7·6–9·5)	20·5 (19·6–21·0)
		Difference	2·9	4·5	6·4	6·9	—

* By subtracting 'chloral hydrate alone' values (see text).

and without liver extract in which both chloride liberation and SH loss have been determined. Again it is seen that the liberation of chloride ions involves a loss of SH. For the experiments in the presence of extract the values for chloride liberated, corrected for spontaneous liberation of chloride ions (i.e. by subtraction of the 'chloral hydrate alone' values), represent the liberation of chloride due to extract + cysteine. By subtracting the SH lost for 'extract + cysteine' from that for 'extract + cysteine + chloral hydrate', values should be obtained which represent the SH loss from extract + cysteine concerned in the liberation of chloride ions from chloral hydrate. This difference, therefore, should be comparable with the corrected values for chloride liberation. The assumption that the loss of SH due to oxidation (or other unknown reactions) is the same in the presence or absence of chloral hydrate is not, however, justified since oxidation would almost certainly be greater in the absence of chloral when there is no competition with the chloride-SH reaction. The difference values would thus be expected to be low, and it may reasonably be concluded that both with and without liver extract there is a loss of SH corresponding fairly closely with the liberation of chloride ions.

Chloral hydrate and other SH *compounds.* Experiments similar to those described above were carried out with other SH compounds. From these only the average values (from at least three experiments) corresponding to the 'differences' in Table 6 have been recorded in Table 7 and only 4 hr. values are given. The loss of SH with glutathione and extract was greater than that expected from the chloride ions liberated. With thioglycollate and extract the SH loss was also somewhat greater than the chloride liberation, although there was no loss of SH when chloride ions were liberated in the absence of extract.

Other chloro compounds with cysteine. With butyl chloral hydrate in the presence of extract the loss in SH was approximately equivalent to chloride liberated, but in the absence of extract the SH loss was greater than that equivalent to the chloride liberated (Table 7). With trichloroacetamide, both with and without extract, the SH loss was much less than would have been expected from the amount of chloride liberated.

Chloral hydrate and cystine. In experiments in which cystine (0·02 M) was added to liver extracts with chloral hydrate there was a significant increase in chloride liberation (1·1 m-equiv./l. digest in 4 hr.) over that obtained from extract and chloral hydrate alone (2·1 m-equiv./l.). There was no increase in the absence of extract. When cystine was added to extract without chloral hydrate it was found that there was almost always an increase in SH content (average 0·3 m-equiv./l. in 4 hr.). This increase may explain the effect of cystine upon the activity of liver extract. In the presence of chloral hydrate there was a loss in SH corresponding to 0·7 m-equiv./l.

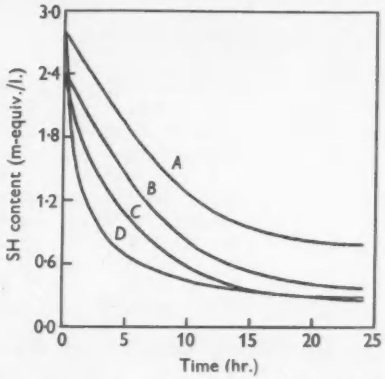

Fig. 4. The effect of previous boiling (1 hr.) upon the SH content of rabbit-liver extract incubated with and without chloral hydrate (0·024 M) at pH 6·8. Each curve represents average results from three experiments. Curve *A*, untreated extract. Curve *B*, boiled extract. Curve *C*, boiled extract with chloral hydrate. Curve *D*, untreated extract with chloral hydrate.

Effect of boiled liver extract. It has already been stated that considerable liberation of chloride ions from chloral hydrate occurs in the presence of boiled extract. The average results for utilization of SH from three experiments are summarized in Fig. 4.

Table 7. *Average liberation of chloride ions from chloro compounds and the corresponding loss in* SH *content due to incubation with* SH *compounds with and without rabbit-liver extract*

(Chloro compound 0·024 M, SH compound 0·02 M, pH 6·8, 37°, 4 hr. incubation. Results expressed as m-equiv./l.)

	Without liver extract		With liver extract	
Substrates	Cl^- liberated	SH loss	Cl^- liberated	SH loss
Chloral hydrate with:				
Cysteine	2·0	1·3	9·0	6·4
Glutathione	1·8	1·7	4·5	6·5
Thioglycollate	3·1	0·0	4·0	4·6
Butyl chloral hydrate with cysteine	1·3	1·7	7·3	6·4
Trichloroacetamide with cysteine	2·5	0·5	1·8	0·5

Boiling causes a reduction in the SH content of the extract, but even with boiled extract there is a more rapid disappearance of SH with chloral hydrate than with boiled extract alone.

Effect of chloral hydrate upon the activity of urease. Urease is an SH enzyme and should be inhibited by a substance such as chloral hydrate which reacts with SH groups. Urease was incubated with chloral hydrate for periods up to 3 hr. prior to determination of the urease activity of the digest. The results recorded in Table 8 show that treatment with chloral hydrate causes considerable inhibition of urease.

chloride ions by tissue extracts. These compounds are either aliphatic, or the chlorine is attached to an aliphatic side chain. In no instance was there appreciable liberation of chloride ions from nuclear-substituted aromatic compounds. It is far from certain, however, that the liberation of chloride from the aliphatic compounds is due to an enzyme such as that claimed by Heppel & Porterfield (1948) to liberate halide from some halogenated hydrocarbons. The greater part of the factor responsible is remarkably stable to heat. Only part of the activity of a liver extract is retained after dialysis. It has been shown that chloride ions can readily be liber-

Table 8. *Effect of preliminary incubation with chloral hydrate upon the activity of urease*

(Results expressed as percentage conversion of urea, 0·9 g./l.)

Exp. no.	Time of preliminary incubation (hr.)	Concn. of chloral hydrate (M)	Percentage of urea converted				
			0·5 hr.	1 hr.	1·5 hr.	2 hr.	2·5 hr.
1	0	0	44	72	86	91	91
		0·024	31	41	57	63	66
2	0	0	41	69	85	91	93
		0·096	23	30	33	34	34
3	1	0	40	69	83	—	92
		0·096	11	16	19	—	20
4	2	0	30	52	67	—	82
		0·096	5	7	8	—	9
5	3	0	26	43	54	61	67
		0·096	3	5	6	7	7

Table 9. *Effect of preliminary incubation for 30 min. with chloro compounds upon the clotting power of papain.*

Exp. no.	Chloro-compound	Amount of papain (mg./10 ml. milk)						
		20	24	28	30	32	36	40
		Reciprocal of clotting time (min.$^{-1}$)						
1	None	0·8	0·9	1·0	1·2	1·5	1·7	—
	Chloral hydrate (0·02 M)	0·5	0·7	0·7	0·9	1·0	1·2	—
2	None	—	1·0	1·5	1·6	1·7	2·1	2·2
	Chloral hydrate (0·04 M)	—	0·7	0·8	0·9	1·1	1·3	1·5
3	None	—	0·8	0·9	1·2	1·3	1·6	1·8
	Butyl chloral hydrate (0·01 M)	—	0·8	0·8	0·9	0·9	1·2	1·5

Effect of chloral hydrate upon the activity of papain. Another SH enzyme, papain, was incubated with chloro-compound for 30 min. previous to determination of activity in clotting of milk. As with urease there was considerable loss of activity. Typical results are shown in Table 9 which records the reciprocal of the time in minutes taken to clot milk using varying amounts of papain.

DISCUSSION

It is clear from the experiments described that a considerable percentage of the chlorine of certain organic chloro compounds may be liberated as ated from chloral hydrate in the absence of liver extract if cysteine is present, and that in the presence of liver extract the addition of cysteine enhances the liberation of chloride ions. The experimental results suggest a quantitative relationship between the chloral hydrate and the SH groups of cysteine and/or liver extract, although such a relationship was not always clear when other chloro or SH compounds were used. It must, however, be remembered that the method of estimation of SH content did not permit the estimation of SH groups bound to protein, and that SH groups may also be used up in reactions, other than atmospheric oxidation, not involving the chlorine atoms, e.g. by condensation

between SH and aldehyde group (cf. Schubert, 1936). Such reactions could account for a loss in the SH content of the digest greater than that expected from the chloride ions liberated, e.g. from chloral hydrate with glutathione or thioglycollic acid (Table 7). It seems probable that the action of liver extract in liberating chloride from chloro compounds is for the most part not enzymic but due to chemical reaction between the chloro compound and SH compounds in the liver extract. If there is a chloride-liberating enzyme it can only play a very small part in the reactions which have been studied. The fact that the addition of the disulphide cystine causes some enchancement of liberation of chloride ions in the presence of liver extract suggests that the extract can reduce disulphide compounds and so enable them to react with chloro compounds.

SUMMARY

1. The effect of incubation with tissue extracts upon the liberation of chloride ions from twenty-nine organic chloro compounds has been examined.

2. Some compounds, including those in which the chlorine is directly attached to the benzene ring, do not liberate appreciable amounts of chloride. Some aliphatic chloro compounds which liberate little chloride, e.g. chloral hydrate, butyl chloral hydrate, or none, e.g. trichloroacetamide, hexachloroethane, spontaneously in aqueous solution, liberate considerable amounts in the presence of liver extract; others which liberate considerable amounts of chloride spontaneously, e.g. benzotrichloride, benzyl chloride, liberate even more in the presence of liver extract.

3. The loss in SH content of the extract always observed when chloride ions are liberated suggests that the reaction is largely one between the chlorine of the chloro compound and the SH of SH compounds.

4. The liberation of chloride ions is enhanced by the addition of SH compounds both in the presence and absence of liver extracts.

5. Previous boiling does not greatly diminish the chloride-liberating activity of liver extracts.

6. It is concluded that the liberation of chloride ions in the presence of liver extract is mainly a reaction between chloro compound and SH compounds which is not enzymic.

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Studies in Detoxication

43. A STUDY OF THE ARYLSULPHATASE ACTIVITY OF TAKADIASTASE TOWARDS SOME PHENOLIC ETHEREAL SULPHATES

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Several types of sulphatases occur in nature. These are distinguished by the nature of the ester group found in the ethereal sulphates which they hydrolyse (Fromageot, 1950). Takadiastase, a commercial preparation of *Aspergillus oryzae*, possesses arylsulphatase (phenolsulphatase) activity (Neuberg & Kurono, 1923), for it hydrolyses phenolic monoesters of sulphuric acid but has no action on aliphatic sulphuric esters. Others preparations of *A. oryzae* such as 'Mylase P' (Abbott, 1947) and 'Clarase' (Dzialoszynski, 1947) are also active. Arylsulphatase also occurs in animal tissues, especially liver, and according to Huggins & Smith (1947) rat neoplasms contain significantly higher amounts of the enzyme than normal tissues.

Mylase P has been shown to release phenolic oestrogens from their conjugates in pregnant mares' urine in yields 20% higher than those obtained by acid hydrolysis (Cohen & Bates, 1949). The use of sulphatase thus provides a gentle method of hydrolysis for ethereal sulphates where acid hydrolysis might prove destructive. We required sources of arylsulphatase for this purpose, and the present work was undertaken to provide possible methods of assay of arylsulphatase and to obtain information about its activity towards different phenolic sulphuric esters.

Recent methods of arylsulphatase assay are those of Abbott (1947) using potassium phenylsulphate with the Folin-Ciocalteu reagent, and of Huggins & Smith (1947) using potassium *p*-nitrophenylsulphate as substrate (cf. Abbott & East, 1949). In the present work we have compared the action of takadiastase on the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids. In each case the liberated phenols were estimated in alkaline solution as their anions whose spectra were different from the corresponding undissociated phenols and ethereal sulphates. By this method each phenol could be estimated spectrophotometrically or colorimetrically in the presence of its ethereal sulphate, using a relatively short incubation time in the enzyme experiments.

EXPERIMENTAL

Spectroscopic measurements were made with a Unicam quartz spectrophotometer, Model SP500, colorimetric measurements with a Hilger Spekker absorptiometer and pH measurements with a Cambridge pH-meter.

Preparation of substrates. The potassium salts of *o*- and *p*-nitro-, *p*-chloro- and *p*-aldehydo-phenylsulphuric acids and the sodium salt of *m*-nitrophenylsulphuric acid were prepared according to Burkhardt & Lapworth (1926) and Burkhardt & Wood (1929). The potassium salts of 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids were prepared for the first time by one of us (Smith, 1951). These salts were purified by recrystallization from water or ethanol-water mixtures until free from inorganic sulphate, chloride and free phenols. Their purity was checked by analysis for Na or K gravimetrically, or for phenols spectroscopically.

Absorption spectra of the substrates and phenolic anions. These were determined in 0·1 N-NaOH and are recorded in Table 1. Fig. 1 shows the spectra of 4-nitrocatechol and its monosulphate and illustrates the difference between the spectrum of a phenolic anion and that of its sulphate.

Preparation of the enzyme solution. Takadiastase (Parke, Davis and Co. Ltd.) was either dissolved in water, or in 0·5 M-acetate buffer as required. These solutions were kept at 0°, but they deteriorated in about a week. The takadiastase used contained added lactose (reducing sugar 96%, calc. as lactose) and 0·4% N (Kjeldahl). This commercial preparation was thus largely lactose. It has been reported that phosphates inhibit sulphatase and therefore phosphate buffers were avoided. At the optimum pH of sulphatase (about pH 6), acetate buffers are not highly efficient, so that they were used at 0·5 M strength to ensure their effectiveness.

The action of takadiastase on p-*aldehydo- and* p-*chloro-phenylsulphates*

Methods

(*a*) *Using potassium* p-*aldehydophenylsulphate.* To each of four tubes there were added 0·4 ml. of 0·5 M-acetate buffer and 0·4 ml. enzyme solution in 0·5 M-acetate buffer. To two of these tubes ('test') there was added 0·4 ml. of a solution of potassium *p*-aldehydophenylsulphate in 0·5 M-acetate buffer of the same pH (all solutions having been previously warmed to 37°). The other two tubes were the controls. All tubes were stoppered and incubated for 1 hr. at 37°. After incubation, 0·4 ml. of substrate solution was added to the

Table 1. *Absorption maxima of certain phenols and their ethereal sulphates in* 0·1 N-NaOH

	$\lambda_{max.}$	$\epsilon_{max.}$	$\lambda_{max.}$	$\epsilon_{max.}$
o-Nitrophenol	283	3850	412–415	4500
Potassium *o*-nitrophenylsulphate	260	4400	—	—
m-Nitrophenol	290	4050	395	1450
Sodium *m*-nitrophenylsulphate	265	6500	—	—
p-Nitrophenol	230	4550	402	17 000
Potassium *p*-nitrophenylsulphate	280	9000	—	—
p-Chlorophenol	222	8900	245 298	11 650 2600
Potassium *p*-chlorophenylsulphate	215	10 500	267·5 275	350 300
p-Hydroxybenzaldehyde	237	7680	330	27 500
Potassium *p*-aldehydophenylsulphate	258	16 300	—	—
Nitroquinol	237	13 100	540	3850
Potassium 4-hydroxy-2-nitrophenylsulphate	235	17 055	380	1390
Potassium 4-hydroxy-3-nitrophenylsulphate	225 279	16 800 3900	423	4750
4-Nitrocatechol	390–395	6300	510	11 300
Potassium 2-hydroxy-5-nitrophenylsulphate	405–410	18 150	—	—

two control tubes and 3·8 ml. of 0·15 N-NaOH to all four tubes. The extinctions of the tubes were then read at 330 mμ. in the spectrophotometer, using 1 cm. cells, against a blank of 0·8 ml. buffer, 0·4 ml. water and 3·8 ml. 0·15 N-NaOH. The amount of *p*-hydroxybenzaldehyde liberated was then calculated from the relation:

$$p\text{-hydroxybenzaldehyde } (\mu g.) = \frac{(E_t - E_c) \times 122{\cdot}1 \times 10^6 \times 5}{27\,000 \times 100}$$
$$= 22{\cdot}2\,(E_t - E_c),$$

where E_t and E_c are the observed spectrophotometer readings of the 'test' and 'control' tubes respectively at 330 mμ.; 122·1 is the molecular weight and 27 000 the $\epsilon_{max.}$

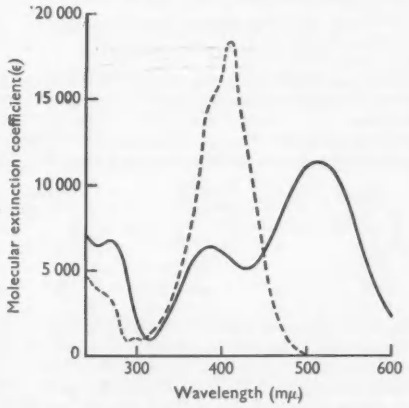


Fig. 1. The ultraviolet absorption spectra of 4-nitrocatechol (full line) and potassium 2-hydroxy-5-nitrophenylsulphate (broken line) in 0·1 N-NaOH.

in alkali of *p*-hydroxybenzaldehyde. Control tubes of substrate and buffer were omitted because a separate experiment showed that no *p*-hydroxybenzaldehyde was liberated on incubating potassium *p*-aldehydophenylsulphate with acetate buffers from pH 4·1 to 6·7. The control tubes containing enzyme and buffer, however, were included because the light absorption of the enzyme extracts may vary slightly on incubation with acetate buffer. At 330 mμ. proteins absorb slightly, but in view of the very low protein content of takadiastase, the removal of protein was unnecessary. However, with preparations of arylsulphatase of high protein content, it may be necessary to remove protein as described in the next section.

(*b*) *Using potassium* p-*chlorophenylsulphate.* The procedure followed was similar to that described by Spencer & Williams (1951) for the assay of β-glucuronidase with *p*-chlorophenylglucuronide. In preliminary experiments it was found that potassium *p*-chlorophenylsulphate on incubation for 3 hr. with 0·5 M-acetate buffers, pH 4·1–6·7, yielded no spectrophotometrically detectable amount of *p*-chlorophenol. The procedure for the assay of the arylsulphatase activity of takadiastase was the following.

The reagents and procedure up to the incubation were as in section (*a*) above except that centrifuge tubes were used. After incubation for 2 or 3 hr., 0·4 ml. of substrate solution was added to the control tubes, and then 4·8 ml. of absolute ethanol to all four tubes. The tubes were centrifuged for 3 min. to remove any precipitated protein and 5 ml. of the clear supernatants transferred to tubes containing 5 ml. 0·2 N-NaOH. The extinctions of these solutions were then read at 245 mμ. in the spectrophotometer against blanks made up of 0·4 ml. acetate buffer and 0·8 ml. water diluted with ethanol and 0·2 N-NaOH as for the test solutions. The amount of *p*-chlorophenol liberated is

$$\frac{(E_t - E_c) \times 128{\cdot}5 \times 6 \times 10\,000}{11\,650 \times 5} = 132(E_t - E_c)\ \mu g.$$

A study of the recovery of *p*-chlorophenol measured at 245 mμ. and of *p*-hydroxybenzaldehyde at 330 mμ., showed that the maxima appeared to obey the Lambert-Beer Law, and that the phenols could be recovered over a wide range of concentrations to the extent of 100 ± 3%.

Effect of pH *and substrate concentration* on the hydrolysis of potassium *p*-aldehydo- and *p*-chloro-phenylsulphate by takadiastase is shown in Table 2.

In optimum conditions with these two substrates, extent of hydrolysis was proportional to time up to 4 hr. and to the quantity of takadiastase present up to 0·5%.

Table 2. *Investigation of effects of* pH *and substrate concentration on hydrolysis of substituted phenylsulphates by takadiastase*

(Temperature 37° throughout; other conditions noted in parentheses; taka = takadiastase.)

Substrate (all K salts unless otherwise stated)	Optimum pH	Optimum substrate concn. and K_m
p-Aldehydophenylsulphate	5·8 (little variation 5·5–6·1) (1% taka; 0·003M-substrate; 1 hr.)	$2·7 \times 10^{-3}$M No inhibition up to 3×10^{-2}M K_m, $2·1 \times 10^{-4}$M (pH 5·8; 1% taka; 1 hr.)
p-Chlorophenylsulphate	6·15 (sharp maximum) (3% taka; 0·0125M-substrate; 3 hr.)	1×10^{-2}M No inhibition up to 3×10^{-2}M K_m, $9·7 \times 10^{-4}$M (pH 6·15; 3% taka; 3 hr.)
o-Nitrophenylsulphate	6·3 (little variation 6·0–6·5) (4% taka; 0·005M-substrate; 1 hr.)	6×10^{-3}M No inhibition up to 2×10^{-2}M K_m, $3·4 \times 10^{-4}$M (pH 6; 4% taka; 1 hr.)
m-Nitrophenylsulphate (Na salt)	6·2–6·4 (little variation 5·9–6·5) (4% taka; 0·005M-substrate; 2 hr.)	6×10^{-3}M No inhibition up to 2×10^{-2}M K_m, $8·35 \times 10^{-4}$M (pH 6; 4% taka; 2 hr.)
p-Nitrophenylsulphate	6·2 (little variation 6·0–6·4) (1% taka; 0·005M-substrate; 1 hr.)	$1·5 \times 10^{-2}$M No inhibition up to 2×10^{-2}M K_m, $1·7 \times 10^{-4}$M (pH 6·1; 1% taka; 1 hr.)
4-Hydroxy-2-nitrophenylsulphate	6·0 (little variation 5·9–6·1) (2% taka; 0·002M-substrate; 2 hr.)	2×10^{-3}M No inhibition up to 5×10^{-3}M K_m, $1·25 \times 10^{-4}$M (pH 5·9; 2% taka; 2 hr.)
2-Hydroxy-5-nitrophenylsulphate	5·8–5·9 (2% taka; 0·0025M-substrate; 2 hr.)	$2·5 \times 10^{-3}$M Inhibition beyond $2·5 \times 10^{-3}$M K_m, $3·5 \times 10^{-4}$M (pH 5·9; 2% taka; 2 hr.)

The action of takadiastase on the o-, m- *and* p-*nitrophenylsulphates*

Method

A solution (1 ml.) of the substrate dissolved in M-acetate buffer and the aqueous enzyme solution (1 ml.) were added to two tubes. These tubes, together with a control tube containing the substrate-buffer solution only, were incubated at 37° for the required period. Then 1 ml. of 0·33N-NaOH was added to all three tubes and 1 ml. of enzyme solution to the control tube. The extinctions of the contents of the first two tubes were then read in the spectrophotometer, with the contents of the control tube in the blank cell of the instrument. Readings were made at 412 mμ. for *o*-, at 395 mμ. for *m*- and at 402 mμ. for *p*-nitrophenol. The amount of nitrophenol in 3 ml. of test solution was then calculated from the relation, μg. nitrophenol = Ex, where x is 92·66 for *o*-, 287·6 for *m*- and 24·5 for *p*-nitrophenol and E is the spectrophotometer reading at the appropriate wavelength, x being calculated as before from known $\epsilon_{max.}$ and molecular weights of the phenols and the dilutions of the solutions. The maxima of each phenol appeared to obey the Lambert-Beer Law.

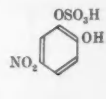
The effects of pH and of substrate concentration on the hydrolysis of the three nitrophenylsulphates are quoted in Table 2. In optimum conditions with these three substrates the extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4% for the *o*- and *m*-substrates and up to 1·2% for the *p*-substrate.

The action of takadiastase on certain hydroxynitrophenylsulphates

The two ethereal sulphates, 4-hydroxy-2-nitro- and 4-hydroxy-3-nitro-phenylsulphuric acids (I and II) give rise to nitroquinol on hydrolysis. Nitroquinol on making alkaline gives a purple colour ($\lambda_{max.}$ 540 mμ., $\epsilon_{max.}$ 3850) which can be estimated with the Spekker absorptiometer. The colour

I

II



III

fades on standing, but is sufficiently stable to allow readings of its intensity to be taken. Similarly, 2-hydroxy-5-nitrophenylsulphuric acid (III) gives rise to 4-nitrocatechol which gives a relatively stable red colour in alkaline solution (λ_{max} 510 mμ., $\epsilon_{max.}$ 11 300) which again can be estimated in the colorimeter. The alkaline nitrocatechol colour is more intense than that of nitroquinol and is therefore more suitable for the assay of arylsulphatase. The standard curves for nitroquinol and nitrocatechol were constructed by dissolving 10–70 μg. of nitroquinol or 4–30 μg. of 4-nitrocatechol in 2 ml. water and adding 0·5 ml. of 2·5N-NaOH. The intensity of colour varied linearly with the amount of the two phenols. The nitroquinol and nitrocatechol colours were read immediately in the Spekker absorptiometer with

Ilford no. 604 (Spectrum green) filters, and using 1 cm. cells holding 0·5 ml. of solution.

Method. The substrate solution (1 ml.), made by dissolving the potassium salt of the ethereal sulphate in M-acetate buffer, was added to each of three tubes. To two of the tubes 1 ml. of the aqueous enzyme solution was added and all three tubes were incubated at 37° for the required time. To each tube there was then added 0·5 ml. of 2·5 N-NaOH and to the third tube (control) 1 ml. of enzyme solution. The colours of the two test solutions were then measured in the Spekker absorptiometer with the control solution in the blank cell of the instrument. The amount of nitroquinol or nitrocatechol liberated was then read from the standard curves.

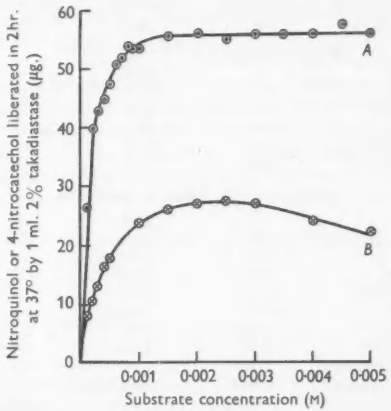


Fig. 2. The effect of substrate concentration on the arylsulphatase activity of takadiastase. *A*, potassium 4-hydroxy-2-nitrophenylsulphate (I) as substrate, where no inhibition by excess substrate up to 0·005 M is seen; and *B*, potassium 2-hydroxy-5-nitrophenylsulphate (III) as substrate, showing inhibition by excess substrate.

The effect of pH and of substrate concentration on the hydrolysis of I and III by takadiastase is shown in Table 2. In the case of III there was an inhibition by excess of substrate which is shown graphically in Fig. 2.

In optimum conditions with I and III extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4%.

In the case of the 4-hydroxy-3-nitrophenylsulphate (II) incubation up to 4 hr. only released 6 μg. of nitroquinol, so that takadiastase appeared to have practically no hydrolytic action towards this ethereal sulphate. This sulphate was also more resistant to acid hydrolysis than its isomer (I), for I is completely hydrolysed by 0·25 N-H_2SO_4 at 98° in less than 10 min., whereas II requires at least 20 min. for complete hydrolysis under the same conditions.

Inhibition of arylsulphatase by 4-hydroxy-3-nitrophenylsulphate

Takadiastase shows very little hydrolytic activity towards potassium 4-hydroxy-3-nitrophenylsulphate (II). This could be due to the inability of the enzyme to combine with II to form the enzyme-substrate complex, or to the formation of a stable enzyme-substrate compound. If the second condition is correct then potassium 4-hydroxy-3-nitrophenylsulphate should behave as an inhibitor of arylsulphatase since it will combine under certain conditions with all the available enzyme and prevent its action on other hydrolysable substrates. The effect of potassium 4-hydroxy-3-nitrophenylsulphate upon the activity of takadiastase towards potassium 2-hydroxy-5-phenylsulphate was therefore studied.

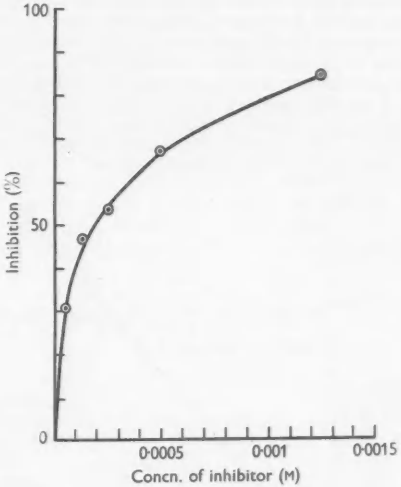


Fig. 3. The inhibition by potassium 4-hydroxy-3-nitrophenylsulphate (II) of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by takadiastase. For conditions, see text.

The hydrolytic action of 0·5 ml. of 2% takadiastase upon 0·0025 M-potassium 2-hydroxy-5-nitrophenylsulphate was measured as above, in the presence of increasing concentrations of potassium 4-hydroxy-3-nitrophenylsulphate. The results (see Fig. 3) showed that the latter substance was highly inhibitory, 50% inhibition being caused by a concentration of about $1{\cdot}4 \times 10^{-4}$ M.

The nature of the inhibition. The activity of 2% takadiastase at increasing concentrations of potassium 2-hydroxy-5-nitrophenylsulphate in the absence and presence of $2{\cdot}5 \times 10^{-4}$ M-potassium 4-hydroxy-3-nitrophenylsulphate was measured at pH 5·8. The plot of $1/v$ against $1/S$ showed an increase in slope (see Fig. 4) in the presence of the inhibitor, whereas the ordinate intercept had the same value as in the absence of the inhibitor, thus indicating competitive inhibition. According to Lineweaver & Burke (1934) the increase in slope $= K_s I/K_I$, where I is the inhibitor concentration and K_I is the dissociation constant of the enzyme-inhibitor complex. In this particular experiment K_s ($= K_m$) was $6{\cdot}8 \times 10^{-4}$ and K_I was calculated to be $2{\cdot}4 \times 10^{-6}$ M. Thus the affinity of potassium 4-hydroxy-3-nitrophenylsulphate for the enzyme was about 300 times ($6{\cdot}8 \times 10^{-4}/2{\cdot}4 \times 10^{-6}$) that of potassium 2-hydroxy-5-nitrophenylsulphate.

Inhibition of arylsulphatase activity by some inorganic compounds

It has been shown that arylsulphatase is inhibited by compounds which react with aldehyde groups, for KCN, K_2SO_3, phenylhydrazine and NH_2OH are inhibitory (Soda, Egami, Koyama & Horigome, 1939; Rozenfeld & Ruchelman, 1940; Dzialoszynski, 1950). Rozenfeld & Ruchelman (1940) concluded that arylsulphatase contained an aldehyde group in its prosthetic group. Furthermore, it has been reported that oxalate, fluoride, sulphate and phosphate ions and heavy metals inhibit the enzyme (Tanaka, 1938; Inouye, 1929; Dziaoszynski, 1950; Huggins & Smith, 1947). According to Tanaka (1938) and Seligman, Chauncey & Nachlas (1951) $MgCl_2$ activates the enzyme, whereas Hommerberg (1931) claims that this compound inhibits slightly. Other workers have obtained activation of the enzyme by $Fe(OH)_3$, $BaCO_3$ and $CaCO_3$. These various workers, however, were working with arylsulphatases from different sources.

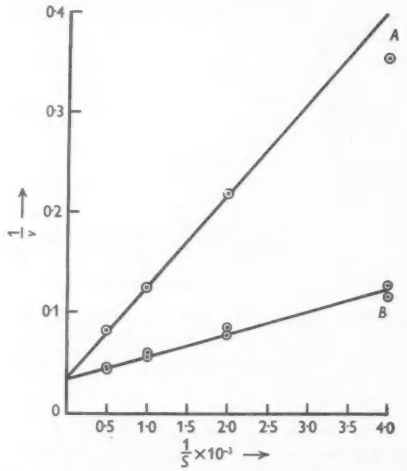


Fig. 4. Competitive inhibition of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by potassium 4-hydroxy-3-nitrophenylsulphate (II). *A*, in the presence of the inhibitor; *B*, in its absence (see text). v = rate of hydrolysis, expressed as μg. 4-nitrocatechol/hr. S = substrate concn. (M).

Using potassium *p*-chloro- and *p*-nitro-phenylsulphates and takadiastase we have examined the action of a number of compounds on arylsulphatase.

Methods

(*a*) *Using* p-*chlorophenylsulphate.* The method described earlier (p. 203) was used, but the 0·4 ml. of substrate solution was replaced by 0·2 ml. inhibitor and 0·2 ml. substrate dissolved in 0·5 M-acetate buffer of pH 6·15. The final concentration of substrate was 0·0125 M. A similar change was made in the control tubes where 0·2 ml. each of inhibitor and substrate replaced the 0·4 ml. of substrate solution.

(*b*) *Using* p-*nitrophenylsulphate.* The inhibitor solutions were made up in M-acetate buffer and adjusted to pH 6·0 with acetic acid or 0·1 N-NaOH as necessary. The inhibitor solution (2 ml.), 0·01 M-substrate solution (2·5 ml.) and 2% takadiastase (0·5 ml.) were mixed in duplicate and incubated for 1·5 hr. at 37°. The control tubes were incubated in the same way using inhibitor and buffer solutions but omitting the enzyme solution until the end of the reaction. The tubes were then all treated with 0·5 ml. of 2 N-NaOH and the liberated *p*-nitrophenol measured.

Table 3. *Effect of inhibitors on arylsulphatase activity of takadiastase*

Compound tested	Final concentration of inhibitor (M)	*p*-Chlorophenol or *p*-nitrophenol liberated (μg.)	Inhibition (%)
p-Chlorophenylsulphate as substrate			
None	—	34·4	—
NaCl	0·02	34·7	0
Na_2SO_4	0·02	34·6	0
$MgCl_2$	0·02	34·5	0
Na_2HPO_4	0·02	27·9	19
Na_2SO_3	0·01	11·0	68
p-Nitrophenylsulphate as substrate			
None	—	20·0	—
KCl	0·02	20·0	0
K_2SO_4	0·02	20·0	0
$MgCl_2$	0·02	14·6	27
Na_2HPO_4	0·02	19·2	4
K_2SO_3	0·02	4·95	75
K_2SO_3	0·002	16·0	20
C_2H_5OH	0·02	18·0	10
NH_2OH, HCl	0·02	2·2	89
NH_2OH, HCl	0·002	7·7	61·5
KCN	0·02	0	100
KCN	0·002	10·7	46·5

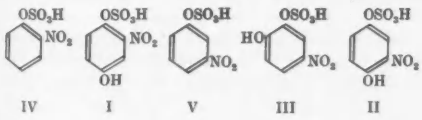
Some of our results are given in Table 3. They confirm that compounds reacting with aldehyde groups (KCN, K_2SO_3, Na_2SO_3 and NH_2OH) inhibit the enzyme. The effect of phosphates was slight but nevertheless evident. $MgCl_2$ (cf. Hommerberg, 1931) seemed to inhibit slightly when *p*-nitrophenylsulphate was used as substrate but not when *p*-chlorophenylsulphate was used.

DISCUSSION

The present work suggests that arylsulphatase can be estimated with almost any phenolic ethereal sulphate hydrolysed by sulphatase at a reasonable velocity provided that the anion of the liberated phenol absorbs light at a different wavelength from the ethereal sulphate. This probably occurs with most phenols. For the most sensitive methods, however, the extinction of the phenolic anion at its wavelength of maximum absorption must be large and of the phenols studied here the *p*-nitro-, *p*-chloro- and *p*-aldehydo-phenols and 4-nitrocatechol seem to satisfy these criteria (see Table 1). The use of *p*-nitrophenol and its sulphate for the assay of sulphatase has already been described (Huggins & Smith, 1947). The convenient maximum absorptions of the *p*-chloro- and *p*-aldehydo-phenol anions

occur in the ultraviolet and are therefore measured with a spectrophotometer covering the ultraviolet. With *p*-nitrophenol, both a colorimeter and a spectrophotometer can be used for measuring the yellow colour of its anion, the latter instrument being more convenient. Since 4-nitrocatechol gives a red colour in alkali, it is readily measured in a colorimeter. We have found the method using 4-nitrocatechol sulphate as substrate readily applicable to the assay of arylsulphatase in animal tissues, the colour being measured with a Spekker absorptiometer. This method is being further developed.

Takadiastase has been shown to hydrolyse the ethereal sulphates of phenol (Neuberg & Kurono, 1923), *p*-cresol (Neuberg & Lindhardt, 1923) *m*- and *p*-chloro-, *p*-bromo-, *o*-, *m*- and *p*-nitro-phenols, quinol, carvacrol, vanillin, *p*-hydroxybenzoic acid, indoxyl, 2-hydroxyquinoline (Neuberg & Wagner, 1925), salicylic acid (Morimoto, 1937), β-naphthol (Noguchi, 1924), oestrogens (Cohen & Bates, 1949), (±)-3-methyl*cyclo*hexylphenol (Weinmann, 1929) and (+)-*p-sec.*-butylphenol (Fromageot, 1929). It failed to hydrolyse the ethereal sulphate of (−)-*p-sec.*-butylphenol (Fromageot, 1929). Our present results cover eight ethereal sulphates of which *o*-, *m*- and *p*-nitro- and *p*-chloro-phenylsulphates have been studied before. Amongst our results we have two examples of the effect on sulphatase activity of the introduction of a hydroxyl group into the aromatic ring of the nitrophenylsulphates. Thus we can compare 4-hydroxy-2-nitrophenylsulphate (I) with *o*-nitrophenylsulphate (IV) and 2-hydroxy-5-nitrophenylsulphate (III) with *m*-nitrophenylsulphate (V). The introduction of an OH into IV and V, to give I and III respectively, causes the optimum pH for sulphatase activity to become more acid (see Table 4). Thus the optimum for IV is pH 6·3 moving to 6·0 in I, whilst the optimum for V is 6·3 moving to 5·9 in III. There is also in both cases a similar change in optimum substrate concentration which is 0·006 M for IV and V and about 0·002 M for I and III. Furthermore, K_m for IV and V is about 2·5 times the K_m for I and III ($K_{mIV}/K_{mI} = 2·7$ and $K_{mV}/K_{mIII} = 2·4$). Thus the introduction of a hydroxyl group into these sulphates increases the affinity of the enzyme for the substrate about 2·5 times.



On these grounds it seemed probable that the lack of hydrolytic activity of takadiastase towards II might be due to the formation of a stable enzyme-substrate complex. That this was actually the case was proved by the fact that II competitively inhibited the action of sulphatase on III. It was calculated that the affinity of the enzyme for II was about 280 times that for III and about 50 times that for I.

Table 4. *Action of the arylsulphatase of takadiastase upon various ethereal sulphates*

Ethereal sulphate	$K_m \times 10^4$ M	Optimum pH	Optimum substrate concentration (M)	Phenol liberated/hr. by 1 g. of takadiastase (μg.)	(μmole)	Phenol liberated/hr. by 1 mg. of the N of the takadiastase preparation (μg.)
o-Nitrophenyl	3·4	6·0–6·5	0·006	1100	7·8	275
m-Nitrophenyl	8·35	5·9–6·5	0·006	1750	12·5	437
p-Nitrophenyl	1·7	6·2	0·015	1272	9·15	318
4-Hydroxy-2-nitrophenyl	1·25	5·9–6·1	0·002	1426	9·2	356
2-Hydroxy-5-nitrophenyl	3·5	5·9	0·0025	837	5·4	209
4-Hydroxy-3-nitrophenyl	(0·024)*	—	—	0	0	—
p-Chlorophenyl	9·7	6·15	0·01	1610	12·5	402
p-Aldehydophenyl	2·1	5·5–6·1	0·0027	960	7·6	240

* K_I measured at pH 5·8.

Our results also enable us to compare the action of sulphatase on three position isomers, namely the *o*-, *m*- and *p*-nitrophenylsulphates. The affinity ($1/K_m$) of the enzyme is greatest for the *p*- and least for the *m*-isomer, the ratio $1/K_{m,p-} : 1/K_{m,o-} : 1/K_{m,m-}$ being 5 : 2·5 : 1.

Although $1/K_m$ is least for *m*-nitrophenylsulphate, more *m*-nitrophenol is liberated per hour by the enzyme from *m*-nitrophenylsulphate than *o*- and *p*-nitrophenols from their corresponding sulphates (Table 4).

The commercial preparation of takadiastase used in this work contained much added lactose and only a small amount of it was mould material. The nitrogen content was 0·4% and the protein content 2·5% (0·4 × 6·25). On the basis of its nitrogen content and the fact that most of the material of the preparation was added lactose, the original dried mould preparation must be a relatively good source of aryl-

sulphatase, for the enzyme in 1 g. of the takadiastase nitrogen would release 32 mg. of *p*-nitrophenol (see Table 4), for example, from the corresponding ethereal sulphate in 1 hr. at 37°.

Our experiments were carried out at 37°, and Abbott (1947) has reported that the optimum temperature of the enzyme is 50°. From Abbott's results arylsulphatase appeared to be about 50% more active at 50° than at 37°.

SUMMARY

1. A study has been made of the action of the arylsulphatase activity of takadiastase towards the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids.

2. The liberated phenols were determined spectrophotometrically or colorimetrically as their anions, which have different spectra from the corresponding ethereal sulphates. The spectra of these sulphates are recorded.

3. Optimum pH's, Michaelis constants and optimum substrate concentrations have been determined for these ethereal sulphates.

4. Potassium 4-hydroxy-3-nitrophenylsulphate was not hydrolysed by takadiastase and inhibited the action of the enzyme towards other ethereal sulphates. This inhibition is competitive and is due to the formation of a stable enzyme-substrate complex.

5. A study has also been made of the inhibition of arylsulphatase by inorganic compounds and aldehyde reagents. It is confirmed that arylsulphatase is inhibited by compounds reacting with aldehydes.

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Seasonal Changes in the Carbohydrates of the Jerusalem Artichoke Tuber

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Several workers (Dubrunfaut, 1867; Colin, 1919; Thaysen, Bakes & Green, 1929; Dedonder, 1951*c*) have noted the change in carbohydrate composition which takes place when the tubers of the Jerusalem artichoke (*Helianthus tuberosus* L.) are stored over the winter. This change may be inferred from the optical rotation of the expressed juice, which changes in the positive direction, and is confirmed by the observations that in the spring less inulin may be obtained from the tubers and more of the carbohydrate is fermentable. Similar observations have been made on chicory, *Cichorium Intybus* L., by Wolff & Geslin (1918).

Bacon & Edelman (1951) suggested that all these changes could be the result of a redistribution of fructose residues among the many carbohydrate

components revealed by paper-partition chromatography of extracts; a relative increase in the proportion of those of lower molecular weight (the smallest of which is sucrose) should give the mixture a more positive rotation (cf. the rotation of these substances: Dedonder, 1951*a*) and the increased fermentability could be due to the more rapid action of yeast invertase on them (Dedonder, 1951*b*).

The following is an account of experiments designed primarily to test this hypothesis. The results also give some indication of the nature of the metabolic changes that take place in hibernating tubers.

METHODS

In order to reduce the actions of enzymes to a minimum the following extraction procedure was adopted, differing in some respects from that described earlier (Bacon & Edelman, 1951):

About 200 ml. of water were brought to the boil in a weighed 500 ml. beaker and kept at the boil while a known weight of tubers (about 100 g.) was added as thin slices cut with a stainless-steel knife. Boiling was continued for a further 3 min., the whole cooled to room temperature, and weighed. The contents of the beaker (usually weighing about 250 g.) were then tipped into a Waring blender and disintegrated without further additions. Samples (about 5 ml.) of this mash were weighed and dried for 24 hr. to determine their content of dry matter.

The mash was then squeezed through madapollam, leaving a small residue, and 50 ml. samples of the filtrate were treated with 5 ml. 30% (w/v) lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$). The precipitate was filtered off by gravity on a Whatman no. 1 filter paper and to 32 ml. of the filtrate were added 5 ml. 3% (w/v) sodium oxalate. If the supernatant fluid showed a precipitate with one more drop of sodium oxalate solution, measured amounts were added until precipitation of lead was complete.

The optical rotation of the resulting filtrate was read in a 2 dm. tube, and samples were taken for estimation of reducing substance (RS) and total RS (Edelman & Bacon, 1951*a*). The remaining filtrate was stored over chloroform in the refrigerator. The samples prepared during summer and autumn slowly deposited a precipitate which was presumably inulin; it dissolved when the sample was heated to 80°. Estimations were sometimes repeated on filtrates which had been stored for as long as 9 months and then heated in this way; they showed that no significant changes in composition had taken place.

For chromatographic analysis the filtrate was applied to sheets of Whatman no. 1 paper. These also were stored for several months without detectable changes in the results of the subsequent development and analysis, which were carried out essentially as described by Bacon & Edelman (1951). The procedure was modified in certain details: (1) the spots for analysis were applied in groups of four, between guide spots, in such a way that all the material for analysis was delivered consecutively from the micrometer syringe, thereby decreasing considerably the error of this measurement; (2) the 'paper blanks' were usually cut from a position below free fructose, and deductions made from the ketose content of particular spots in proportion to the area of paper they occupied. The recovery of ketose from the chromatograms was in most analyses between 95 and 100%, and never less than 90%.

MATERIALS

The artichokes used were those grown during 1949, and described by Bacon & Edelman (1951). Small tubers of uniform size and regular shape were planted 1·5 ft. (50 cm.) apart in the same plot in the western outskirts of Sheffield, about 550 ft. (170 m.) above sea level. These were the source of most of the material analysed. A few tubers of similar size and shape, lifted at the same time, were planted at Telham, about 2 miles (3 km.) south-east of Battle, Sussex, at an elevation of about 400 ft. (120 m.). In both situations the plants made considerable vegetative growth, but only in Sussex were flowers produced.

At intervals of about 2 weeks a whole plant was lifted, the tubers taken to the laboratory as quickly as possible, and extracted within an hour. All the tubers were given a preliminary washing under the cold tap, dried with filter paper and weighed. Two 100 g. samples of whole tubers were taken, and washed more thoroughly before extraction. The yield of tubers from single plants varied considerably not only in number and weight but also in shape. No systematic attempt was made to choose representative samples from each plant.

The tubers were not removed from the ground in the autumn; they began to grow again in 1951 rather later in the spring than in 1950.

RESULTS

Calculations from analyses. For the samples taken in August and September the dry weight of the tubers was determined directly by drying slices, but in October and afterwards it was calculated from the dry weight of the mash, assuming no losses of dry matter during the boiling, and complete mixing of the added water with the pulp. For some samples both methods were used.

The free and total RS/100 g. fresh weight of tuber were calculated from the analyses of the madapollam filtrate, neglecting the small residue left on the cloth. It was assumed that the additions of lead acetate and sodium oxalate solutions diluted the resulting filtrates to a volume equal to the sum of the volumes mixed.

The ketose as percentage total ketose recovered of each spot in the paper chromatogram cannot be assumed to represent exactly the distribution of fructose in the various components, nor can the absolute amounts of most of these components be calculated, since, with the exceptions of fructose and sucrose, the pure substances were not available.

Changes in composition with age

Tables 1 and 2 summarize the results of analyses on tubers from 20 July 1950, when they were almost indistinguishable from roots, until May 1951, when shoots 25 cm. long had developed on some of them.

Table 1. *Dry matter of tubers and total reducing substances, ketose and optical rotation of tuber extracts*

(Figures marked * are for a single extraction, all other figures are for duplicate extractions. All extracts were made on the date recorded except for those marked † which were made (30 September 1950) 2 days, (4 December 1950) 7 days, (14 April 1951) 3 days, and (9 June 1950) 3 days later, respectively. ‡ tubers from Sussex.)

Date dug up	Dry matter (g./100 g. fresh tuber)	Total RS (g./100 g. fresh tuber)	$[\alpha]_D$ calculated on total RS	Ketose as % total RS
20 July 1950	12·4*	4·7*	−10·4*	85*
22 Aug. 1950	15·5	11·4	−22·5	95
28 Aug. 1950	16·2	10·0	−20·1	98
4 Sept. 1950	15·6	11·0	−21·0	96
11 Sept. 1950	15·9	11·6	−22·4	95
18 Sept. 1950	17·0	13·3	−21·1	—
25 Sept. 1950	16·8	11·8	−19·8	94*
30 Sept. 1950†‡	13·9	9·9	−23·3	97*
3 Oct. 1950	18·4	12·7	−19·4	—
9 Oct. 1950	18·9	14·3	−17·5	—
16 Oct. 1950	18·1	13·7	−16·1	—
23 Oct. 1950	19·4	14·1	−16·3	93*
30 Oct. 1950	20·4	14·8	−16·0	94
6 Nov. 1950	20·7*	15·3*	−9·5	89
9 Nov. 1950	21·5	16·0	−7·5	90*
14 Nov. 1950	20·8	14·5	−4·0	86
20 Nov. 1950	20·8	15·5	−4·9	—
26 Nov. 1950	21·0	15·3	−1·8	—
4 Dec. 1950	20·8	14·0	−1·2	90*
4 Dec. 1950†	33·5	23·9	−3·0	—
19 Dec. 1950	21·1	14·2	+2·0	88
2 Jan. 1951	21·5	15·2	+3·0	—
17 Jan. 1951	20·0	14·0	+5·4	79*
12 Feb. 1951	19·9	13·6	+4·1	—
5 Mar. 1951	20·1	15·6	+0·5	—
2 Apr. 1951	22·0	13·7	+3·6	84
14 Apr. 1951†‡	22·1	16·8	+8·1	77
25 May 1951	15·2	10·9	−16·6	88*
9 June 1950†	10·8	6·3	−9·2	81

Table 2. *Distribution of ketose among the components of tuber carbohydrate*

(Results refer to the same extracts as those in Table 1.)

Date dug up	$[\alpha]_D$ calculated on total RS	Ketose as % of ketose recovered from chromatogram					Recovery of ketose (%)
		Spot *F*	Spot 1	Spot 2	Spot 3	Spot 4	
30 July 1950	−10·4	—	4·3	5·1	6·1	—	102
22 Aug. 1950	−22·4	—	1·8	1·8	2·5	3·0	98
22 Aug. 1950	−22·6	—	1·8	1·8	2·5	3·0	102
28 Aug. 1950	−18·8	—	2·0	2·2	2·8	3·1	101
28 Aug. 1950	−21·4	—	2·2	2·4	2·8	3·1	99
30 Sept. 1950	−24·9	—	1·4	1·7	2·0	2·7	98
23 Oct. 1950	−16·8	—	2·7	3·0	3·5	4·2	100
9 Nov. 1950	−6·9	—	5·7	4·9	6·2	6·1	95
4 Dec. 1950	−0·7	—	6·8	6·3	8·4	8·2	99
17 Jan. 1951	+6·5	—	10·1	8·0	10·7	9·2	98
2 Apr. 1951	+3·3	—	9·1	7·7	7·2	9·7	95
2 Apr. 1951	+4·0	—	9·5	8·0	10·7	10·2	93
14 Apr. 1951	+7·7	1·8	13·7	10·9	12·3	11·2	97
25 May 1951	−19·0	9·6	7·9	4·7	5·5	5·6	—
25 May 1951	−14·2	7·5	7·0	4·6	5·8	6·6	96
9 June 1950	−7·4	25·6	18·6	8·7	7·8	6·8	92

(These tables may be compared with Tables 1 and 4, Bacon & Edelman, 1951.) To them have been added analyses of some old tubers still present in actively growing plants during June 1950; these tubers were still turgid, and conform to the trends in composition which were evident during May of the following year.

The dry weight as percentage of fresh weight increased during the period of sub-aerial growth, but thereafter remained almost constant until the tubers began to sprout, when it declined sharply. There was a considerable increase in the total weight of tuber per plant; in July the total weight was 14 g., in August about 200 g., and in September,

October and November never less than 300 g. and often 600 g. or more. The increase was due partly to increase in the number of tubers, but more to increase in their size. In all plants there was very considerable disparity in size between tubers, the largest ones weighing more than 100 g., the smallest 5 g. or less. Despite this variation the dry weights of duplicate samples agreed to within 10 % in practically all cases.

Total RS as a percentage of the fresh weight of the tubers also showed an increase until the end of October when it remained more or less steady until growth began in April. It is not possible with the data available to estimate the changes in total carbohydrate taking place in a single tuber between November and April, but in view of the small changes in dry weight and total RS as proportions of the fresh weight of tuber it is reasonable to suppose that this change also must have been small and could not exceed 10 %.

Free RS was very small during the period from July to April, constituting 1 % or less of the total RS. Larger amounts found in the Sussex tubers in April (3·8 % of total RS) and in the Yorkshire tubers in May 1951 (10·9 %), and June 1950 (27·8 %), were mainly accounted for as free fructose (see Table 2); there was no appreciable free glucose in any of the extracts examined.

Ketose as percentage of total RS decreased noticeably, displaying differences similar to those recorded by Bacon & Edelman (1951; Table 4); in Yorkshire from 95 to 96 % in August it decreased to 85 % in March. The method of estimation used for ketose is not as satisfactory as that for reducing substances, but even if allowance is made for this, the decrease implies an increase in non-ketose RS (glucose) of about three- to four-fold.

This increase in the proportion of glucose was accompanied by an increase in components with greater R_F values. As already noted by Bacon & Edelman (1951), the fructose was distributed fairly evenly among these components; in particular there was no marked change in spot 1 relative to spots 2–4. The sum of the amounts of ketose in spots 1–4 as a percentage of the total ketose estimated, which may be taken as a measure of the proportion of components of lower molecular weight, increased in Yorkshire from only 9 % in August to 38 % in April, and in Sussex from 8 % in September to 48 % in April, the greatest change taking place in the 2 months following the death of the foliage in the last week of October.

Parallel with this increase in the proportion of components of lower molecular weight there took place a change of optical rotation in the positive direction (Fig. 1; a line has been drawn to emphasize the consistent trend shown during October, November and December, despite the fact that analyses were made on individual plants). In August the rotation, expressed as $[\alpha]_D$ on the basis of the total reducing substances, was $-22{\cdot}5°$; by April it had risen to $+3{\cdot}5$. Again, the most rapid rate of change took place following the death of the foliage. The close connexion between rotation and distribution of ketose in spots 1 to n is shown by Fig. 2, in which the ketose in spots 1–4 as a percent-

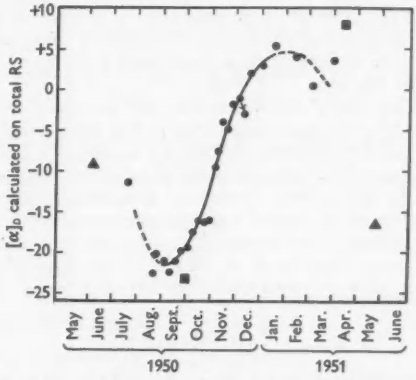

Fig. 1. Seasonal changes in the optical rotation of extracts, plotted from the data in Table 1. Yorkshire-grown tubers: ●, free RS small; ▲, free RS large. Sussex-grown tubers, ■.

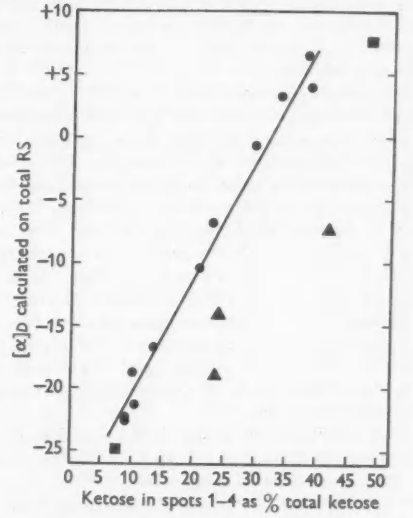

Fig. 2. Relation between the optical rotation of extracts and the sum of the ketose contents of spots 1–4 as percentage total ketose recovered, plotted from the data in Table 2. Yorkshire-grown tubers: ●, free RS small; ▲, free RS large. Sussex-grown tubers, ■.

age of the total ketose estimated is plotted against the $[\alpha]_D$ calculated from the total RS; all the points for extracts containing little free RS lie on or near a straight line.

The single extract made in July 1950 resembled the extracts obtained in November as far as $[\alpha]_D$ and the ketose distribution were concerned. It differed from them in its low content of total RS.

That the changes observed are not simply the result of the rise in temperature following removal from cold ground was shown by analyses of tubers dug up in December and kept at laboratory temperature for a week (Table 1, 4 December 1950). Although the tubers lost considerable amounts of water the $[\alpha]_D$'s of the extracts were not significantly different from those of tubers of the same plant extracted immediately after removal from the ground.

DISCUSSION

It has been shown by Bacon & Edelman (1951) and by Dedonder (1950) that the carbohydrates of the artichoke tuber are more numerous than had been thought previously, and both have suggested that they constitute a series of non-reducing substances which may be considered as being formed from sucrose by the addition of one or more fructofuranose residues.

The results presented here show that in the living plant the relative amounts of these substances change in a consistent way during growth, dormancy, and germination of the tubers. This change is accompanied by a change in the optical rotation of the deproteinized extract which has a linear relationship to it, thus strengthening the idea that each 'spot' on the paper chromatogram represents a single substance. It is significant that the single extract made in July 1950 conforms to the relationship established with later samples, because although it has a low total RS content (as would be expected from the results for August and September), its $[\alpha]_D$ and distribution of ketose resemble those of November samples.

Dedonder (1951*b*) has described the action of yeast invertase on spots 1–4 (*A*, *B*, *C*, *D* in his terminology); from this it might be expected that the juice from tubers harvested in the spring would be fermented more readily by yeast, as has been observed before (Thaysen *et al.* 1929).

Certain suggestions as to the nature of the metabolic changes occurring in the tubers may be made on the basis of the present results. The most striking change is the increase in relative glucose content, shown by estimation of ketose as percentage of total RS, and also by the increase in the proportion of the spots 1–4, which have relatively high glucose contents. Such a change could be achieved by the action of the hydrolytic enzyme found in extracts (Edelman & Bacon, 1951*a*) followed by utilization of the fructose produced, but this would involve the disappearance of about three-quarters of the fructose present in October. Such an explanation would seem to be excluded by the indirect evidence available. Colin (1919) rejected the idea that the inulase of Green (1888) was responsible for the changes that he observed.

An alternative explanation is that a slow conversion of fructose to glucose takes place (cf. Dedonder, 1951*d*), probably not involving the formation of free sugars, and that from this glucose more sucrose is produced. Then by the transfructosidation reaction described by Edelman & Bacon (1951*b*) a redistribution of fructose residues occurs, increasing the amounts of the substances of smaller molecular size at the expense of those of large.

This would imply that during the period of carbohydrate storage the tendency is for substances of high molecular weight (and hence of low glucose content) to be formed. The analysis of the July tubers possibly represents the earliest stage of storage, when the amount of polysaccharide laid down is still small.

The large amount of free fructose found in sprouting tubers could perhaps be due to a very greatly increased activity of the hydrolytic enzyme system during the extraction procedure, but more probably indicates the manner in which carbohydrate is mobilized. (In unpublished experiments we found no increase in the units of hydrolytic enzyme extractable from stored tubers during the period 8 March to 9 May 1949.) The decrease in proportion of spots 1–4 probably indicates that glucose is also being liberated, perhaps by hydrolysis of sucrose.

It is not easy to see why the composition of the tubers should change in this way; it may represent a reversal of the process by which polysaccharide is laid down during the period of photosynthesis. Similar changes would presumably take place if the tubers were harvested and stored in a suitably cool and not too dry place.

SUMMARY

1. Analyses of dry matter, and of the ketose and combined reducing substances in aqueous extracts, have been made on tubers of the Jerusalem artichoke during the period June 1950 to May 1951, confirming the occurrence of a change in carbohydrate composition during the winter months, already noted by several investigators.

2. A change in the positive direction of the optical rotation of extracts has been correlated with an increase in the proportion of carbohydrate com-

ponents of lower molecular weight estimated by quantitative paper-partition chromatography.

3. The significance of these findings is discussed in relation to the structures proposed by Dedonder (1951*a*) and Bacon & Edelman (1951) for the tuber carbohydrates and a suggestion is made as to the nature of their metabolic interconversion.

We are very grateful to Dr R. Dedonder for sending us a copy of his Thesis on 'Les glucides du topinambour', which contains much experimental detail not yet published.

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Studies in Carotenogenesis

4. NITROGEN METABOLISM AND CAROTENE SYNTHESIS IN *PHYCOMYCES BLAKESLEEANUS*

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Preliminary experiments on the relationship between nitrogen metabolism and carotenogenesis in *Phycomyces blakesleeanus* (Garton, Goodwin & Lijinsky, 1951) suggested that carotene was not synthesized when well formed mycelial mats were transferred to a medium containing glucose but no assimilable nitrogen. The present paper records a full investigation into this aspect of the problem of carotene formation in *Phycomyces*.

Part of this work has already been briefly described (Goodwin, Lijinsky & Willmer, 1951).

EXPERIMENTAL

The general cultural conditions, the technique of mycelial transfer, and the analytical methods for dry weight, lipid, and carotene determinations have previously been described in detail (Garton *et al.* 1951; Goodwin & Lijinsky, 1951). Nitrogen determinations were carried out using the micro-Kjeldhal method of Markham (1942).

RESULTS

Carotene production and nitrogen assimilation in growing cultures

Mycelia of *Phycomyces* cultured on media containing 0·2% (w/v) of L-asparagine and either 2·5, 1·5 or 1·0% (w/v) of glucose (unless otherwise stated the salt and aneurin concentrations are those used by Garton *et al.* 1951) were harvested at various times after inoculation and analysed for dry weight, lipid, carotene and nitrogen. The results obtained using the medium containing 2·5% glucose are recorded in Fig. 1. From this figure it will be seen

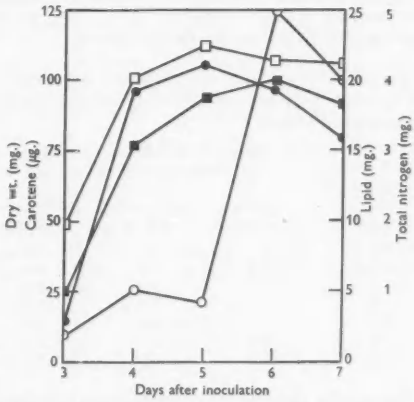

Fig. 1. The dry weight, lipid, carotene and nitrogen content of developing *Phycomyces* cultured in an 8 oz. medicine bottle on 15 ml. of a standard medium (Garton *et al.* 1951) containing 2·5% (w/v) glucose and 0·2% (w/v) asparagine. □—□, dry weight; ○—○, carotene; ■—■, lipid; ●—●, nitrogen.

that, assessed by cessation of either dry weight production or nitrogen assimilation, growth must be complete before carotene synthesis proceeds at all

rapidly. Growth is complete within 4 or 5 days, but at this time carotene formation is only about 16% of the final amount, which is rapidly synthesized within the next 2–3 days. Very similar results, which are not recorded here, were obtained using the medium containing 1·5% (w/v) of glucose, except that the amount of carotene synthesized was not as great. As would be expected from the previous results of Garton *et al.* (1951), after growth had ceased no further carotene was produced on the medium containing 1% of glucose owing to the absence of dissimilable glucose.

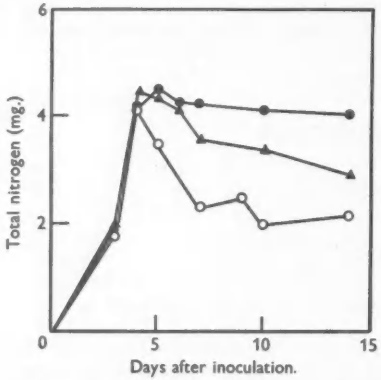

Fig. 2. The nitrogen content of developing *Phycomyces* cultured on media containing 0·2% (w/v) of L-asparagine but varying amounts of glucose. ○—○, 1% glucose; ▲—▲, 1·25% glucose; ●—●, 2% glucose.

Table 1. *Total nitrogen concentration in mycelia of* Phycomyces *grown on media containing varying amounts of glucose*

Age of culture (days)	Nitrogen concentration (%) in medium containing 1% (w/v) glucose	1·5% (w/v) glucose	2·5% (w/v) glucose
3	6·8	8·7	7·9
4	12·3	6·1	5·4
5	5·17	4·3	4·5
6	5·8	5·3	4·3
7	4·3	4·8	4·6
10	4·9	5·0	3·7
14	5·2	4·8	4·0

The results obtained for the nitrogen uptake on the different media are worthy of separate consideration. Fig. 2 shows that the maximal nitrogen content occurs 3–4 days after inoculation, and is the same irrespective of the glucose concentration of the medium. In the case of mycelia grown on media containing excess glucose (2·5%, w/v) most of this nitrogen is retained in the mycelia as they age. If, however, the media contain insufficient glucose it is clear from Fig. 2 that, as the mycelia mature, a considerable amount of the nitrogen is lost to the medium. Because dry weight production is directly proportional to, whilst the nitrogen uptake is independent of, the glucose concentration of the medium, young mycelia growing on a glucose-poor medium have a very high nitrogen concentration. This can be seen from Table 1 which also demonstrates that owing to the different rates at which nitrogen is lost from the mycelia, its concentration in all mycelia tends to be the same after about 7 days' growth. In older cultures, the nitrogen concentration of the mycelia grown on the glucose-rich medium again tends to be lower; this is due to the maintenance of the mycelial weight in old cultures when sufficient glucose is present. On the glucose-poor media old cultures tend to decrease in dry weight.

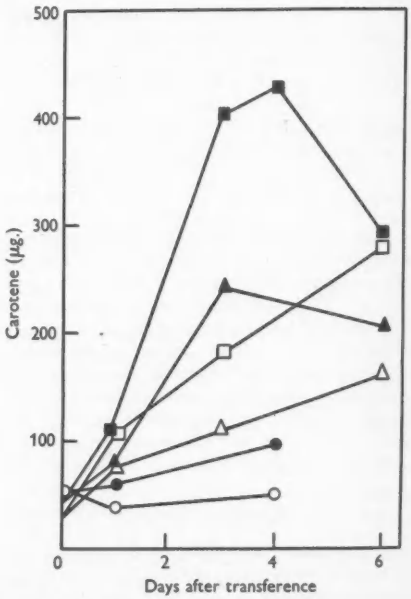

Fig. 3. The carotene content of mycelia after transference, when 4 days old, to hard media containing 2·5% glucose and either: ■—■, 0·2% (w/v) L-asparagine (unbuffered medium); ▲—▲, no nitrogen (unbuffered medium); ●—●, 0·2% (w/v) L-asparagine (buffered medium, pH 7); ○—○, no nitrogen (buffered medium, pH 7); □—□, 0·2% (w/v) L-asparagine (medium with high salt concn. see Table 2); △—△, no nitrogen (medium with high salt concn.).

Carotene synthesis in the presence and absence of assimilable nitrogen

The results just described suggest that the failure of Garton *et al.* (1951) to observe carotene synthesis by well developed mycelial mats dissimilating

glucose in the absence of nitrogen was due to a factor other than the absence of nitrogen from the medium. The only cultural difference in the transference experiments of Garton *et al.* (1951) compared with the other experiments described by them was that the formed mycelia were transferred to the standard medium buffered at pH 7·0; all other media were unbuffered. A series of experiments was thus undertaken to test whether the buffering of the medium inhibited carotenogenesis under conditions known to be normally favourable to synthesis. As the buffering of the medium necessitated a considerable increase in salt concentration of the medium, the effect of increasing this, irrespective of buffering power, was also investigated. The concentrations of the salts used to prepare the various media are recorded in Table 2 and the experimental results in Fig. 3 and Table 3. Fig. 3 shows that carotene synthesis on an unbuffered medium with a normal salt concentration is considerable, whether the medium contains nitrogen or not. On buffered media, on the other hand, no carotene is synthesized on the nitrogen-free media (there is actually a slight loss of carotene during the first day after transference), whilst only a very small amount is synthesized when nitrogen is present. On media with only slight buffering capacity but with twice the salt concentration of the buffered media, i.e. ten times the normal salt concentration, carotenogenesis still takes place, but to a somewhat lesser degree compared with the standard medium. Table 3 shows that dry weight production is not appreciably affected by the ionic condition of the medium; lipogenesis, on the other hand, is stimu-

Table 2. *Salt concentration in media used to investigate the effect of buffering and salt concentration on carotene synthesis*

	Concentration of salt % (w/v)				
Constituent	Standard medium	Medium buffered to pH 7·0	Unbuffered medium with salt concn. equal to twice that of buffered media	Medium buffered to pH 6·0	Medium buffered to pH 5·2
$MgSO_4.7H_2O$	0·05	0·05	0·5	0·05	0·05
KH_2PO_4	0·15	0·363	1·5	0·817	0·885
Na_2HPO_4	0·00	0·568	0·00	0·094	0·0237

Table 3. *Amounts of dry weight, lipid and carotene produced by well formed (4-day-old) mats of* Phycomyces *after transference to buffered and unbuffered media containing 3·0 % (w/v) of glucose and either no nitrogen or 0·2 % (w/v) of* L*-asparagine as the sole nitrogen source*

(The salt concentrations of the various media are given in Table 2. The mats were grown on filter paper in Petri dishes (7·5 cm. diameter) containing 45 ml. of medium and transferred to similar dishes containing the same amount of medium (see Goodwin & Lijinsky, 1951).)

Time after transference (days)	With nitrogen				Without nitrogen			
	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)
				Standard medium				
1	383	79	20·6	210	113	68	60·2	500
3	387	74	19·1	1060	103	35	34	2070
5	294	45	15·3	1070	84	21	25	2410
				Medium buffered at pH 7·0				
1	142	42	29·6	—	34	18	52·9	0
2	331	110	33·2	45	101	41	40·6	0
3	356	122	34·2	73	72	39	54·1	0
4	341	125	36·6	111	—	—	—	0
5	382	98	34·6	74	71	32	45·1	0
				Medium with high salt concentration (unbuffered)				
1	237	70	29·5	250	(−17)	(−2)	—	—
3	317	87	27·5	420	—	15	25·4	1110
5	369	112	30·4	630	80	80	37·5	1380

Table 4. *Dry weight and lipid production of* Phycomyces *cultured on the standard medium (containing 3 % (w/v) glucose) buffered at various* pH *values*

Age of culture (days)	Medium buffered at pH 7·0 Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Medium buffered at pH 6·0 Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Medium buffered at pH 5·2 Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Unbuffered medium Dry wt. (mg.)	Lipid (mg.)	Lipid (%)
4	57·5	17·4	30·1	48·5	—	25·6	59·1	17·0	28·8	63·5	17·5	27·5
7	103·0	34·7	33·7	109·2	36·4	33·3	98·3	28·5	28·9	83·7	14·3	17·1
11	104·8	33·6	32·0	104·9	26·3	25·0	97·7	21·7	22·1	69·7	10·7	15·3
17	93·4	14·1	15·0	91·8	16·8	18·3	96·5	18·2	19·0	—	—	—

lated on media containing a high salt concentration probably irrespective of pH (see later). Lipogenesis was also stimulated in the absence of nitrogen; up to 50 % of the dry weight formed by a well developed mat, after transference to a medium not containing nitrogen and buffered at pH 7·0, is lipid.

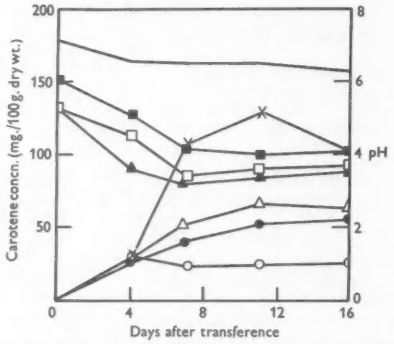

Fig. 4. The concentration of carotene in mycelia grown on standard medium buffered at various pH values; ○—○, medium buffered at pH 7·0; ●—●, medium buffered at pH 6·0; △—△, medium buffered at pH 5·2; ×—×, unbuffered medium.

Also the corresponding changes in the pH of the media during growth; ——, medium initially buffered at 7·0; ■—■, medium initially buffered at 6·0; □—□, medium initially buffered at 5·2; ▲—▲, unbuffered medium.

On unbuffered media the synthesis of carotene by developed mats is relatively greater in the absence of nitrogen (Table 3), for the concentration of carotene in the dry matter produced by a mat dissimilating glucose in the absence of nitrogen is about twice that observed when nitrogen is present in the medium.

The results of a series of experiments carried out to see if the effect of buffering the medium, just described for transferred cultures, is observed in ordinary cultures are recorded in Table 4 and Fig. 4. Three buffered media were used at pH 7·0, 6·0 and 5·2 (see Table 2 for the salt concentration). It will be seen from Fig. 4 that the pH of the medium buffered at 7·0 remained almost constant throughout the experiment at about 6·5 (the pH of the medium after inoculation with spore suspension), whilst those of the other buffered media fell to 4·1 and 3·8 respectively; the final pH of the control (unbuffered) medium was 3·2. Carotene synthesis varied inversely with the pH of the medium. The total dry weight production was not affected by the pH of the medium, whilst, as in the case of transferred cultures, lipogenesis was affected in exactly the opposite way to carotenogenesis, being considerably higher in media of high pH.

DISCUSSION

The results recorded in Fig. 1 show that, in the presence of excess glucose and a source of readily assimilable nitrogen, the major portion of carotene synthesis of *Phycomyces* occurs after growth (dry weight production and/or nitrogen assimilation) is complete. It appears, then, that most of the carotene is synthesized from the products of the dissimilation of glucose, quite probably under anaerobic conditions, for carotene accumulates in the surface of the mycelium in contact with the medium and, as the cultures are static, conditions in this region must be almost completely anaerobic. Carotenogenesis in *Phycomyces* could thus be an example of fermentative assimilation, a type of synthesis known to occur in yeasts (Clifton, 1946).

The question naturally arises as to how the observation of Goodwin & Lijinsky (1951), that, in the presence of suboptimal amounts of glucose, L-valine and L-leucine stimulate carotenogenesis, fits in with these just reported. It will be seen from Fig. 1 that a small amount of carotene is always synthesized as the mycelium is growing, thus indicating that the enzyme systems necessary for this synthesis are present in growing mycelia. If the amino-acid being used as the nitrogen source yields on deamination a carbon residue readily incorporated into the carotene molecule, then obviously the amount of carotene synthesized as the fungus is growing will be increased; this is what occurs with L-valine and L-leucine.

There is now no doubt that the inability of Garton *et al.* (1951) to observe carotenogenesis in *Phycomyces* mats dissimilating glucose in the absence of nitrogen

was due to their media being buffered at about pH 7. The present results show that in any medium in which the pH is not allowed to fall to 3·2, carotene synthesis is considerably inhibited. A probable explanation of this is that, assuming that the pigments are produced by fermentative assimilation, the changes in pH alter the pathway of glucose dissimilation, and the production of the building units required for carotene synthesis is reduced. The classic example of this is the very sharp optimal pH for the production of citric acid by *Aspergillus niger*, and the increased production of acids other than citric at other pH values (Bernhauer, 1926). The reduced synthesis by mats transferred to unbuffered media with a high salt concentration (Fig. 3) is probably due to the slight buffering power of this medium rather than to any osmotic effects, for it was noted that during the experiment the pH of these media never fell below 3·8. The pH of normal media falls to 3·2 (Fig. 4).

The present work has demonstrated that lipid production in *Phycomyces* depends also on the pH of the medium, the higher the pH the greater the lipogenesis. Again, this can be explained on the assumption of variation in the products of glucose dissimilation according to the pH of the medium. Similar variations in lipid production have been observed in *A. niger* (Pontillon, 1930) and *A. fisheri* (Prill, Wenck & Peterson, 1935).

Nitrogen metabolism

The observations reported here on the assimilation of nitrogen by *Phycomyces* fall into line with those by Steinberg (1939) on *A. niger*, i.e. the amount of nitrogen removed from the medium is very much the same regardless of the glucose concentration of the medium. The present work further shows that on media containing only a low concentration of glucose a considerable part of the nitrogen assimilated during the early stages of growth is rapidly lost as the mycelia age, probably owing to the utilization of nitrogen-containing material for the production of energy. On media containing adequate amounts of glucose little or no excretion takes place.

SUMMARY

1. The major portion of the carotene synthesized by *Phycomyces blakesleeanus*, using a readily assimilable source of nitrogen which does not also provide a specific carotene precursor, is produced only after the mycelial mat is fully grown, as measured by dry weight production and/or nitrogen assimilation.

2. Media buffered at high pH values (5·2–7·0) support normal growth of *Phycomyces*, although carotenogenesis is almost completely inhibited. Lipogenesis, on the other hand, is stimulated under these conditions.

3. Well formed mats of *Phycomyces* dissimilating glucose can synthesize relatively more carotene in the absence of assimilable nitrogen than in its presence. Under these conditions lipid synthesis is also relatively greater on the nitrogen-free media.

4. Nitrogen uptake by *Phycomyces* is independent of the glucose concentration of the medium. When the glucose concentration of the medium is low a considerable amount of the assimilated nitrogen is lost as the mycelia age.

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The Estimation of Acetone Bodies

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In a study of the pathogenesis of bovine ketosis it was felt desirable to be able to follow in some detail the changes in all three ketone bodies (acetone, acetoacetic acid and β-hydroxybutyric acid) which have been shown by various workers to occur in excessive quantities in blood, milk and urine during the course of that disorder. Hitherto, little or no attempt has been made at complete differentiation as both acetone and acetoacetic acid are usually determined in the one fraction. Moreover, *iso*-propanol which we have found (Robertson, Thin & Stirling, 1950) to be present in this disease and in pregnancy toxaemia of ewes, has been unsuspected hitherto in such conditions and, as it is oxidized to acetone by chromic acid, its presence will undoubtedly have influenced the estimation of total ketones by various methods. In this paper the term 'Total Acetone Bodies' is used, therefore, to include acetone, acetoacetic acid, β-hydroxybutyric acid and *iso*-propanol.

The accurate determination of free acetone in the presence of acetoacetic acid is difficult. Van Slyke's method, using Denigès reagent (Van Slyke, 1917, 1929) which has frequently been utilized in ketosis studies, cannot be used to estimate free acetone; in addition, it is not very sensitive as we have found that no precipitate is formed with small amounts of acetone bodies of the order of 5 mg./100 ml. or less. Moreover, using this method, *iso*propanol appears to be oxidized to acetone partly in the acetone plus acetoacetic acid fraction, and partly in the β-hydroxybutyric acid fraction, so making the accurate determination of the individual acetone bodies impossible.

Attempts at using the distillation methods of Shaffer & Marriott (1915) and Behr (1928, 1940) for free acetone by distillation under reduced pressure, or by blowing a fine stream of air through the solution at room temperature, in order to prevent acetoacetic acid decomposing, gave poor results, as with quantities likely to be present in biological fluids, only a small proportion of the acetone could be recovered. The method of Werch (1940, 1941), utilizing diffusion into Nessler's solution in Conway microdiffusion units, though providing a very delicate qualitative test, did not give accurate quantitative results; the time for the appearance of the precipitates varied and they could not be estimated gravimetrically with any degree of success. The following method was therefore devised, combining with some modification the oxidation technique of Greenberg & Lester (1944), the diffusion method of Werch (1940, 1941) and Seifert (1948), and the colorimetric technique of Behr & Benedict (1926). It depends on the development of an orange to red coloration when an alkaline solution of salicylic aldehyde is left in the presence of acetone, the depth of colour formed being directly proportional to the amount of acetone present. This can be used to estimate free acetone, the acetone formed by hydrolysis of acetoacetic acid, and that formed by chromic acid oxidation of β-hydroxybutyric acid and of *iso*propanol.

METHODS AND RESULTS

Apparatus. Microrefluxing apparatus (Greenberg & Lester, 1944); Conway microdiffusion units.

Reagents. Approx. 0·15 N-$Ba(OH)_2$ and approx. 2·5% (w/v) $ZnSO_4.7H_2O$ (Greenberg & Lester, 1944); 20 N-H_2SO_4; 10% (w/v) $K_2Cr_2O_7$; 1·5% (w/v) $K_2Cr_2O_7$ in 15·6 N-H_2SO_4; 20% (w/v) acetic acid; 4 N-KOH; salicylic aldehyde supplied by British Drug Houses Ltd.

Colour reagent. To 1 ml. of salicylic aldehyde are added 8 ml. of 4 N-KOH and the solution well mixed; 2 ml. are used for each estimation.

Preparation of standard graphs. A standard solution of acetone (3 ml.), prepared according to Behr & Benedict (1926) and containing 0–10 mg. acetone/100 ml. of solution are placed in the outer chamber of a Conway dish with a few drops of 20% (w/v) acetic acid; 2 ml. of the colour reagent are placed in the inner chamber. The lid is put on firmly, after greasing the rim, and the dish is left 0·5–3 hr. in an incubator at 37°, or any other standard time and temperature that is convenient; alternatively, it may be kept at room temperature overnight, when the maximum colour is obtained regardless of temperature. It should not be left standing, however, more than 20 hr. as the colour reagent tends to blacken after this interval. A blank is run using water instead of the standard solution.

After the requisite time 0·5 ml. of the coloured solution in the central chamber is added to 2 ml. of water in a test tube, and the resulting solution read in the Spekker photoelectric colorimeter against the blank similarly prepared, using 2 ml. cells and a blue-green filter (Ilford 603).

Fig. 1 shows a graph prepared by estimating known amounts of acetone. It will be seen that Beer's Law is strictly adhered to over the range 0–10 mg./100 ml. Table 1 shows that results with standard acetone solutions are reproducible within ±4%.

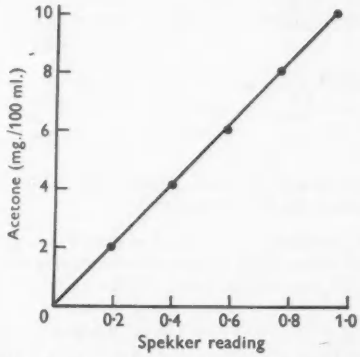

Fig. 1. Standard curve for acetone, measuring the colour produced with alkaline salicylic aldehyde against a blank after standing 2 hr. at 37°. Filter: blue-green, Ilford 603.

Table 1. *Reproducibility using standard solutions of acetone*

Time interval (hr.)	Theoretical result (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
2	7·92	7·80	−1·5
2	7·92	8·00	+1·0
Overnight	7·92	7·70	−2·8
Overnight	7·92	8·10	+2·3
2	1·98	1·90	−4·0
2	1·98	2·03	+2·5
Overnight	1·98	2·00	+1·0
Overnight	1·98	2·05	+3·5

APPLICATION TO BIOLOGICAL MATERIAL

Blood

Protein precipitation: to 2 ml. of distilled water in a centrifuge tube 1 ml. of blood is added and the solution well mixed; 3 ml. of 0·15 N-$Ba(OH)_2$ are then added, followed by 3 ml. of 2·5% (w/v) $ZnSO_4.7H_2O$. The solution is well mixed, the tube stoppered and centrifuged. The clear supernatant liquid is used for estimating acetone, acetone plus acetoacetic acid and 'Total Acetone Bodies'.

Free acetone. A 3 ml. sample of the supernatant fluid is placed in the outer chamber of a greased Conway dish and the estimation carried out as above. Owing to dilution during precipitation and oxidation, it is necessary to multiply the results obtained from the standard graphs by the factor 9 to get the results in mg. acetone/100 ml. of original solution.

Free acetone can also be estimated without precipitation of protein if so desired. Recovery experiments which were carried out using the complete technique of precipitation etc., on standard solutions in water and blood showed the same limits of accuracy as in direct estimation of standard solutions (Table 2).

Table 2. *Recovery of added acetone*

(The blank was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
Standard acetone solution		
7·92	7·80	−1·5
7·92	8·00	+1·0
7·92	8·10	+2·3
Standard solutions in blood		
7·92	8·01	+1·1
7·92	7·83	−1·1
7·92	8·10	+2·3
15·84	16·20	+2·3
Standard solutions in blood (direct method)		
7·92	8·00	+1·0
7·92	8·10	+2·3
7·92	8·20	+3·5
15·84	15·50	−2·1

Acetoacetic acid. Acetoacetic acid is determined by difference, the free acetone determined as above being subtracted from the acetone plus acetoacetic acid value, obtained as follows.

Acetone plus acetoacetic acid. A 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus and 1·1 ml. of 20 N-H_2SO_4 and a glass bead are added. The apparatus is shut firmly after applying a very thin film of grease to the ground-glass joint, the flow of water is started and the contents of the reflux tube boiled for 5 min. The apparatus is then cooled rapidly under running water, tipped to mix well, and 3 ml. of the solution are transferred to the outer chamber of a Conway dish for the determination of the acetone present. The factor in this case is 12·3.

Recovery experiments were carried out as before, using commercial grade ethyl acetoacetate purified by distillation under reduced pressure. The results obtained (Table 3) came within ±4% of the theoretical amounts.

Table 3. *Recovery of acetoacetic acid, expressed as acetone*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Standard solutions			
5·09	5·04	0	−0·9
5·09	5·04	0	−0·9
10·18	10·10	0	−0·8
10·18	9·84	0	−3·3
19·57	19·68	0	+0·6
19·57	19·68	0	+0·6
Standard solutions in blood			
5·09	5·16	0	−1·4
5·09	5·04	0	−0·9
5·76	18·45	12·92	−4·0
5·76	18·45	12·92	−4·0
11·52	24·60	12·92	+1·4
11·52	24·60	12·92	+1·4

Table 4. *Recovery of* iso*propanol, expressed as acetone*

Amount added (mg./100 ml.)	Microdistillation: Amount found (mg./100 ml.)	Microdistillation: Blank (mg./100 ml.)	Microdistillation: Error (%)	Method of Friedmann (1938): Amount found (mg./100 ml.)	Method of Friedmann (1938): Blank (mg./100 ml.)	Method of Friedmann (1938): Error (%)
			Standard solution			
7·43	7·38	0	−0·6	7·25	0	−2·4
7·43	7·38	0	−0·6	7·50	0	+0·9
7·43	7·38	0	−0·6	7·25	0	−2·4
			Standard solution in blood			
7·43	11·81	4·31	+0·9	7·5	0	+0·9
7·43	11·81	4·31	+0·9	7·25	0	−2·4
7·43	11·44	4·31	−4·0	7·25	0	−2·4

iso*Propanol.* *iso*Propanol is oxidized to acetone under the conditions normally employed for estimating 'Total Acetone Bodies' and so is included in this term. It can also be estimated separately using a modification of Friedmann's (1938) method in which the *iso*propanol alone is oxidized to acetone. A Markham steam distillation apparatus is used in this procedure, except in the case of blood, as it is more convenient for small quantities, as well as being quicker and easier to clean between samples.

A 50 ml. sample of blood is transferred to a large conical flask with 100 ml. of distilled water, 10 ml. of $HgSO_4$ solution (Friedmann, 1938) are added and the solution mixed, then 15 ml. of 10% (w/v) sodium tungstate and a little wax to prevent frothing. The solution is well shaken and steam-distilled, about 100–200 ml. of distillate being collected.

The distillate is washed into a round-bottomed flask, 5 ml. of $HgSO_4$ solution added and excess 10% (w/v) $Ca(OH)_2$ suspension till the solution is alkaline. This solution is distilled directly into a conical flask, about 100 ml. of distillate being collected. 10 ml. of 20 N-H_2SO_4 and excess $K_2Cr_2O_7$ are added to the distillate and the flask, loosely corked, is either placed on a drying oven (about 50°) for 2 hr., or left overnight at room temperature.

The solution is then washed into a round-bottomed flask and about 50 g. $MgSO_4$ added. The solution is distilled direct, a little less than 100 ml. distillate being collected; this is made up to 100 ml. with distilled water in a graduated flask and the acetone content determined by placing 3 ml. of the resulting solution in the outer chamber of a Conway dish and proceeding as above, using the factor 5 for the calculation.

Recovery experiments are shown in Table 4. The microrefluxing method used gave results similar to those obtained with Friedmann's method.

β-Hydroxybutyric acid. β-Hydroxybutyric acid is determined by difference, the acetone plus acetoacetic acid plus *iso*propanol values being subtracted from the value for 'Total Acetone Bodies', obtained as follows: a 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus with 0·7 ml. of 1·5% (w/v) $K_2Cr_2O_7$ in 15·6 N-H_2SO_4, and a glass bead. The apparatus is closed firmly as before, the water flow started, and the contents boiled for 10 min. The solution is taken off the boil and 0·4 ml. of 10% (w/v) $K_2Cr_2O_7$ run down the cold finger into the solution by means of a syringe. The apparatus is closed firmly again and the solution boiled for a further 10 min., cooled, tipped to mix the contents and 3 ml. transferred to the outer chamber of a Conway dish and the acetone content determined. The factor here is 12·3.

The accuracy of the method for 'Total Acetone Bodies' when done on acetone and acetoacetic acid solutions was within ± 3% (Table 5). Estimations

Table 5. *Recovery of 'Total Acetone Bodies'*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
	Acetone in standard solutions		
9·50	9·64	0	+1·5
9·50	9·32	0	−1·9
9·50	9·32	0	−1·9
23·76	23·37	0	−1·6
23·76	23·37	0	−1·6
23·76	23·99	0	+0·9
	Acetone in blood		
9·50	12·97	3·20	+2·8
9·50	12·42	3·20	−2·9
9·50	12·67	2·95	+2·3
23·76	27·06	3·20	+0·4
23·76	27·68	3·20	+3·0
23·76	26·45	2·58	+0·4
	Acetoacetic acid in standard solutions		
5·76	5·78	0	+0·3
11·52	11·56	0	+0·3
	Acetoacetic acid in blood		
5·76	27·48	21·89	−3·0
11·52	33·33	21·89	−0·7

of β-hydroxybutyric acid in the quality obtainable, namely, British Drug Houses Laboratory Reagent, gave a practically consistent error of approx. 40% in both standard solutions and in blood (Table 6). Various modifications of this method which were tried, such as altering the concentration of chromic acid, varying the time of oxidation and varying the concentration of β-hydroxybutyric acid, all failed to give any increase in the percentage recovery. It was thought that part of this discrepancy might be due to impurity, but at our request Messrs British Drug Houses Ltd. kindly examined their product which we had used, the sodium salt of β-hydroxybutyric

acid, and reported that it gave the expected 78 % yield of acetone when examined by the method of Greenberg & Lester (1944) and that an approximate determination of purity by precipitation of the sodium with hydrochloric acid in ethanol, followed by the removal of the acid into ether, gave a result of about 98 %. A determination of the sodium content from sulphated ash also gave results equivalent to 98 % purity.

Table 6. *Recovery of β-hydroxybutyric acid, expressed as acetone*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
	Standard solutions		
5·08	3·07	0	39·6
5·08	3·07	0	39·6
5·08	3·08	0	39·4
10·15	6·13	0	39·6
10·15	6·13	0	39·6
10·15	6·26	0	38·3
15·24	9·10	0	40·3
15·24	9·10	0	40·3
15·24	9·35	0	38·6
	Standard solutions in blood		
5·08	7·38	4·31	39·6
5·08	5·41	2·34	39·6
5·08	5·29	2·34	41·9
10·15	9·84	3·73	39·8
10·15	9·84	3·73	39·8
10·15	10·08	3·73	38·3
15·24	13·16	3·73	38·1
15·24	12·79	3·73	40·5
15·24	13·16	3·73	38·1

From our results it would appear, therefore, that chromic acid only oxidizes 60 % of the β-hydroxybutyric acid present to acetone under the above experimental conditions, as contrasted with the 78 % yield of acetone obtained by Greenberg & Lester (1944) using rather different conditions. As our method of determination yields results consistently 40 % low, then to determine the true amount of β-hydroxybutyric acid in a sample the value obtained by subtraction must be multiplied by 5/3. This corrected value added to the previously determined acetone plus acetoacetic acid plus *iso*propanol value will then give the true amount of 'Total Acetone Bodies' present.

Rumen contents

The rumen contents are strained through surgical gauze to remove large pieces of food, and the moderately clear liquid is used for the estimations. The protein is precipitated as for blood and the procedure and factors are the same except for *iso*propanol, where the factor is 5 and the procedure as follows: a 10 ml. sample of the filtered rumen liquor is placed in a Markham steam-distillation apparatus with 2 ml. of $HgSO_4$ solution (Friedmann, 1938) and 2 ml. 10% (w/v) sodium tungstate solution, and steam distilled, about 100 ml. of distillate being collected. This is repeated with a further 10 ml. of liquid and the distillates added.

The combined distillates are washed into a round-bottomed flask and 5 ml. of $HgSO_4$ solution and excess 10% (w/v) $Ca(OH)_2$ suspension added. This solution is distilled direct and the procedure then follows that given for blood.

Milk

As milk contains many reducing substances it is necessary to dilute the solution still further in order to get full oxidation of β-hydroxybutyric acid and *iso*propanol to acetone. This is achieved by precipitating the protein as follows: a 0·4 ml. sample of milk is added to 4·6 ml. of distilled water, 2 ml. of 0·15 N-$Ba(OH)_2$ solution and 2 ml. of 2·5% (w/v) $ZnSO_4.7H_2O$ are added. The tube is stoppered after mixing the contents and centrifuged as before.

The technique employed is the same as for blood except in the case of *iso*propanol where the method is similar to that used for rumen contents.

The factors are, for free acetone 22·5, for acetone plus acetoacetic acid and 'Total Acetone Bodies' 30·75 and for *iso*propanol 5.

Urine

The procedure and factors are the same as for blood except in the case of *iso*propanol, where the rumen technique is used, and the factor is 5.

In certain acute cases of acetonaemia, the content of 'Total Acetone Bodies' in urine rises to several hundred mg./100 ml. In these cases it is advisable to dilute the protein-free liquid still further before oxidation.

Recovery experiments carried out on milk and urine using added acetone gave the results shown in Table 7.

Table 7. *Recovery of added acetone from milk and urine*

(The blank value was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
	Milk	
7·92	7·88	−0·5
7·92	7·88	−0·5
7·92	7·88	−0·5
15·84	16·20	+2·3
15·84	16·20	+2·3
	Urine	
7·92	7·83	−1·1
7·92	7·83	−1·1
7·92	8·10	+2·3
15·84	15·93	+0·5
15·84	15·75	−0·5

Interference

The following substances were tested for interference in the estimation of acetone bodies: acetic acid, lactic acid, sodium chloride, cholesterol, urea, formaldehyde and acetaldehyde. With the exception of acetaldehyde, the results obtained showed there was no interference. This compound in concentrations as low as 3 mg./100 ml. reacts with the colour

reagent to give a slightly opaque orange solution. The interference is lessened a little after boiling with chromic acid, but not sufficiently to allow of an accurate determination even of total ketones in the presence of such amounts of this material.

Recovery from mixed solutions

From the preceding tables it can be seen that using standard solutions of one of the 'Acetone Bodies', the errors lay within the range ± 5 % except in the case of β-hydroxybutyric acid where the percentage yield of acetone was only 60, a discrepancy which is overcome by the use of the appropriate factor.

Experiments were carried out with similar small errors on standard mixtures of 'Acetone Bodies' after complete precipitation and oxidation (Table 8).

Estimations were also carried out on standard mixtures in blood and in rumen contents (Table 9), and the experimental errors were again found to lie within the same range. However, the concurrent experiments carried out using Denigès' method gave percentage errors with a much wider range.

DISCUSSION

From the results it can be seen that the method is readily applicable to two significant biological materials, namely, blood and rumen contents, in acetonaemia and pregnancy toxaemia. It can equally well be used for milk and urine with no appreciable change in precipitation and oxidation technique.

Although by no means perfect it is the first relatively simple method of obtaining a fairly adequate and reasonably accurate differentiation of the various ketone bodies involved in ruminant

Table 8. *Recovery of mixtures of ketone bodies using standard solutions*

Exp. no.	Mixture	Theoretical result (mg./100 ml.)	Amount found (mg./100 ml.)	Error (%)
1	Acetone	7·92	7·75	−2·1
	Acetoacetic acid	3·81	3·68	−3·4
	*iso*Propanol	7·43	7·50	+0·9
	β-Hydroxybutyric acid	5·08	5·30	+4·3
2	Acetone	7·92	7·75	−2·1
	Acetoacetic acid	3·81	3·81	0
	*iso*Propanol	7·43	7·15	−3·7
	β-Hydroxybutyric acid	10·15	9·99	−1·6
3	Acetone	15·84	15·30	−3·4
	Acetoacetic acid	7·62	7·45	−2·2
	*iso*Propanol	14·86	14·00	−5·7
	β-Hydroxybutyric acid	15·45	14·60	−5·5

Table 9. *Recovery of mixtures*

Exp. no.	Mixture	Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Amount recovered (mg./100 ml.)	Error (%)
			In blood			
1	Acetone	7·92	7·87	0	7·87	−0·6
	Acetoacetic acid	3·81	3·63	0	3·63	−5·8
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	5·08	9·60	4·30	5·30	+4·3
2	Acetone	7·92	8·03	0	8·03	+1·0
	Acetoacetic acid	3·81	3·53	0	3·53	−7·0
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	10·15	18·90	8·20	10·70	+5·4
3	Acetone	15·84	15·30	0	15·30	−3·4
	Acetoacetic acid	7·62	7·46	0	7·46	−0·2
	*iso*Propanol	14·86	15·00	0	15·00	+0·9
	β-Hydroxybutyric acid	15·45	23·17	7·18	15·99	+3·5
			In rumen contents			
4	Acetone	7·92	7·87	0	7·87	−0·6
	Acetoacetic acid	3·81	3·93	0	3·93	+3·2
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	5·08	8·08	3·17	4·91	−3·3
5	Acetone	15·84	15·50	0	15·50	−2·1
	Acetoacetic acid	7·62	7·10	0	7·10	−6·8
	*iso*Propanol	14·86	14·40	0	14·40	−3·1
	β-Hydroxybutyric acid	15·45	18·50	3·03	15·47	+0·1

pathology and should prove of value in throwing further light on the pathogenesis of various conditions in which acetonaemia is a prominent feature.

SUMMARY

1. A method has been devised for the estimation as acetone of the individual ketone bodies—acetone, acetoacetic acid, β-hydroxybutyric acid and *iso*propanol—within the range 0–120 mg. acetone/100 ml.

2. The basis of the method is the diffusion of acetone into an alkaline solution of salicylic aldehyde with the production of an orange-red colour, the intensity of which is measured in a photoelectric colorimeter.

3. The application of the method to biological materials such as blood, milk, urine and rumen liquor is described.

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The Preparation and Properties of β-Glucuronidase

3. FRACTIONATION AND ACTIVITY OF HOMOGENATES IN ISOTONIC MEDIA

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The first paper in this series (Kerr & Levvy, 1951) dealt with the fractionation of glucuronidase activity in water homogenates of mouse liver and other organs which is brought about by buffering to slightly acid pH. Changes in the glucuronidase activity of a tissue with the state of proliferation (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950) were seen to be confined to the non-precipitable fraction. In the second paper (Walker & Levvy, 1951), it was shown that an identical fractionation of mouse-liver glucuronidase in untreated water homogenates could be achieved on the high-speed centrifuge, and that the sedimentable fraction was associated with cytoplasmic granules of all sizes. The use of water in preparing tissue homogenates caused pronounced osmotic swelling of the nuclei and other subcellular particles, leading sometimes to disruption. Homogenizing in isotonic media tends to preserve these bodies, and the present paper deals with the partition of glucuronidase activity between sedimentable material and the suspension fluid after homogenizing mouse liver in isotonic sodium chloride or sucrose solution. It was found that liver preparations did not display their full glucuronidase activity until a considerable proportion of the enzyme had been brought into solution.

METHODS

Preparation and fractionation of the enzyme. Unless stated to the contrary, the mouse liver was ground for 1 min. in the glass homogenizer (average speed 5000 rev./min.) with sufficient water, 0·17 M-NaCl or 0·25 M-sucrose solution to give a final tissue concentration of 10%, and samples were taken for assay. Following the procedure already described (Walker & Levvy, 1951) fractionation was then carried out either by centrifuging at a high speed (25000 *g* for 15 min.), or by adjusting the pH to 5·2 with acetate or citrate buffer (final concentration 0·1 N) and centrifuging at a low speed (1500 *g* for 15 min.). In the majority of experiments, the figure for sedimented enzyme activity was found by difference from the figures for total and unsedimented enzyme, the assumption being made that the latter was uniformly distributed between supernatant and precipitate. Control experiments to test this assumption, similar to those carried out with water homogenates (Walker & Levvy, 1951), showed that it also held for the homogenates in isotonic media.

Enzyme assay. After homogenizing liver in isotonic media, the enzyme associated with the subcellular particles did not display its full potential activity (see below). Unless otherwise stated, therefore, such homogenates were treated prior to assay by shaking with Ballotini, grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10 min. When the sedimented material from these homogenates was subjected to direct assay, it was found sufficient to rehomogenize in water.

The enzyme preparations, fractionated or unfractionated, were usually diluted to a final volume of 40 ml./g. moist tissue, and 0·5 ml. was transferred to a tube containing 0·5 ml. 0·01 M-phenolphthalein glucuronide solution (Talalay *et al.* 1946) and 3 ml. 0·1 N-acetate or citrate buffer, pH 5·2. The same buffer was used in the assay as had been used in fractionation. The remainder of the procedure followed that described by Kerr & Levvy (1951). Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μg. phenolphthalein in 1 hr. at 37°.

Microscopy. As before (Walker & Levvy, 1951), preparations were examined under phase contrast.

RESULTS

Fractionation of homogenates in isotonic media

Table 1 shows typical results obtained for the fractionation of glucuronidase activity in adult and infant mouse liver homogenized in water, 0·17M-sodium chloride and 0·25M-sucrose solution. The figures for normal adult were all obtained with samples of the same liver, whilst the infant figures refer to litter mates. Fractionation was carried out with acetate buffer, and in these particular experiments the figures for total, soluble and insoluble enzyme activity were all obtained by direct measurement. The results illustrate the following points. With either adult or infant liver, the total activities for all three types of preparation were in agreement. In each individual case, the figure for the total agreed within experimental error with the sum of the figures for the two fractions. There was, however, a striking difference between water homogenates on the one hand and homogenates in sodium chloride or sucrose solution on the other in the partition of enzyme activity between the two fractions.

Table 1. *Fractionation of mouse-liver glucuronidase activity with acetate buffer after homogenizing in water or in isotonic solution*

(Insoluble enzyme activity found by direct measurement.)

Homogenizing fluid	G.U.*/g. liver		
	Total	Soluble	Insoluble†
Normal adult:			
Water	2900	1740	1140
0·17M-NaCl	2920	280	2600
0·25M-Sucrose	2850	220	2530
Infant (4 days old):			
Water	4810	3600	1240
0·17M-NaCl	4800	780	4020
0·25M-Sucrose	4720	720	4080

* For definition of G.U. see 'Enzyme assay'.

† Washed once on centrifuge with one-tenth volume of 0·1M-acetate buffer; wash fluid added to soluble fraction.

In water the greater part of the activity was in a soluble form, but in the two isotonic media the soluble enzyme only accounted for 10–15% of the total activity. As already noted (Kerr & Levvy, 1951), the activity of the soluble fraction in a water homogenate of infant liver was higher than that in adult by an amount corresponding to the overall increase in activity. This was certainly not the case for homogenates in isotonic media, although the activity of the soluble fraction showed some increase in infants as compared with adults. Sodium chloride and sucrose did not act in these experiments as precipitating agents for enzyme soluble in water alone, but produced their effect by preserving some form of union between the enzyme and insoluble cell debris during homogenization. This was shown by experiments in which homogenates in water were subsequently made 0·17 M with sodium chloride or 0·25 M with sucrose. The partition of the enzyme remained characteristic of a water homogenate.

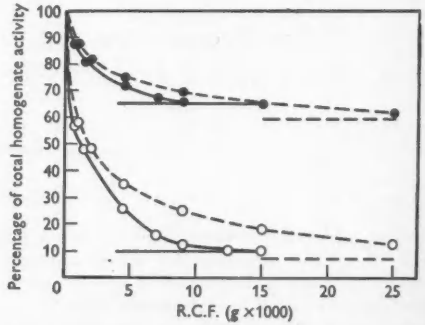

Fig. 1. Supernatant glucuronidase activity after centrifuging unbuffered homogenates of normal adult mouse liver at varying speeds for 15 min. Results expressed as percentages of the initial homogenate activity. ○—○, liver homogenized in 0·17M-NaCl; ●—●, same liver homogenized in water and then made 0·17 M with NaCl; ○- - -○, liver homogenized in 0·25M-sucrose; ●- - -●, same liver homogenized in water and then made 0·25M with sucrose. The horizontal lines indicate the values for acetate soluble enzyme in the various preparations.

As with water homogenates (Walker & Levvy, 1951), the fractionation of glucuronidase activity produced by centrifuging at a high-speed unbuffered homogenates in isotonic media was identical to that produced by acetate buffer. This is shown in Fig. 1 for adult mouse liver. As a control for the effect of the homogenizing medium on the rate of sedimentation of subcellular particles, the tissue was also homogenized in water alone, and sodium chloride or sucrose was added before centrifuging. In each preparation, sedimentation on the high-speed centrifuge ceased when the figure for acetate

soluble enzyme was reached. At this stage, subsequent addition of acetate buffer to the supernatant caused no further separation of enzyme. As already noted (Walker & Levvy, 1951), sodium chloride caused agglutination and more rapid sedimentation of subcellular particles. Sucrose did not produce this effect. Considering either isotonic medium separately, it can be seen that the completeness of sedimentation of the insoluble enzyme at any given relative centrifugal force (R.C.F.) value was the same as in the appropriate control.

In spite of the higher overall glucuronidase activities of infant liver and liver regenerating after partial hepatectomy (Levvy *et al.* 1948), the sedimentation of the insoluble enzyme in homogenates in either isotonic medium followed the same course as that shown in Fig. 1 for the normal adult tissue.

Microscopic observations

Sucrose homogenates. The components of a homogenate of mouse liver in 0·25 M-sucrose resembled closely in appearance those described in similar preparations of rat liver (Hogeboom, Schneider & Pallade, 1948). The nuclei were smooth in outline and structureless, and granules of all sizes were discrete, uniformly dense and spherical. Complete sedimentation of insoluble enzyme was associated with the removal of all but a very few of the smallest visible particles from the suspension. Buffering the homogenate to pH 5·2 with acetate led to agglutination of all the visible components, and consequently much greater ease of sedimentation (cf. Walker & Levvy, 1951).

Saline homogenates. Compared with their appearance in sucrose homogenates, the nuclei in homogenates in 0·17 M-sodium chloride were small, dark and granular, with well marked nucleoli. The granules were similar in appearance to those in sucrose homogenates, but were loosely agglutinated.

Water homogenates. As already noted (Walker & Levvy, 1951), the nuclei and granules showed pronounced osmotic swelling. The former were pale with faint nucleoli and were sometimes vacuolated. The latter were discrete, spherical and vesicular, with much less contrast than the granules in sucrose or saline homogenates.

On progressive dilution with water, the nuclei and granules in a saline or sucrose homogenate took on the appearance of those seen in water homogenates, without perceptible fragmentation of the larger granules. On making a water homogenate isotonic with sodium chloride or sucrose, however, the particles did not revert completely to the appearance typical of a homogenate prepared in the isotonic medium. Most of the granules became smaller and darker, but irregular in outline, while the nuclei became slightly crenated.

Factors influencing the partition of enzyme activity in the homogenate

It would appear from the experiments described above that the greater part at least of the soluble glucuronidase activity in a water homogenate of mouse liver is not originally free in the cytoplasm of the intact cell, but is liberated from the insoluble particles during the preparation of the homogenate. The effects were studied of three variables which might conceivably influence the final partition of enzyme activity after grinding the tissue in the glass homogenizer, namely, the temperature of the homogenizer, the period of homogenizing and the tonicity of the medium.

Temperature. The freshly dissected, chilled tissue was homogenized with cold 0·25 M-sucrose solution for 1 min. in a glass homogenizer surrounded with crushed ice. The centrifuge tubes were also surrounded with ice during the subsequent fractionation using the acetate buffer method. Results obtained in this way were identical with those obtained for another sample of the same liver put through the same treatment without temperature control. This was also true for a third sample of liver homogenized at 37° and then fractionated in the usual way.

Period of homogenizing. Portions of adult liver were homogenized for varying periods, without temperature control, and fractionated by buffering with acetate. The results reveal a progressive release of enzyme from the particles to the solution as homogenizing was prolonged (Table 2). The time factor was least marked in water. There was no inactivation of the enzyme in these experiments.

Table 2. *Fractionation of adult mouse-liver glucuronidase activity after grinding in the glass homogenizer for varying periods with water,* 0·17 M-NaCl *or* 0·25M-*sucrose solution*

Period of homogenizing (min.)	Acetate-soluble enzyme (% total activity)		
	Water	0·17 M-NaCl	0·25 M-Sucrose
0·5	64	9	7
1	66	11	8
2	70	15	15
3	73	19	20
5	75	26	25

An attempt was made to reduce the figure for soluble enzyme activity in an isotonic preparation below the lowest given in Table 2. Liver was ground in a chilled mortar with 0·25 M-sucrose solution and strained through bolting silk. The preparation thus obtained was very similar in its microscopic appearance to the usual sucrose homogenate, and had the same percentage soluble enzyme activity as the 0·5 min. homogenate (Table 2).

Tonicity. Fig. 2 shows the partition of enzyme activity after homogenizing adult liver for 1 min. in varying concentrations of sucrose. No change in the value for acetate-soluble enzyme was observed in passing from the isotonic to hypertonic medium, but as the medium was made increasingly hypotonic there was a progressive rise in the activity of this fraction until the figure for water was reached. The overall glucuronidase activity of the preparation did not vary with the concentration of sucrose.

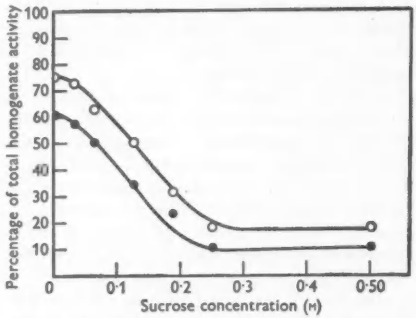

Fig. 2. Acetate-soluble glucuronidase activity in mouse-liver preparations after homogenizing in varying concentrations of sucrose. Results expressed as percentages of the total homogenate activity. ●—●, adult; ○—○, 4-day-old.

Fig. 2 also shows that at all concentrations of sucrose, infant liver had a greater proportion of the enzyme in a soluble form than the adult tissue. The difference became more marked with decreasing concentration of sucrose till in water alone, as already noted, it accounted entirely for the higher overall activity of the infant tissue. Similar curves were obtained for liver homogenized in sodium chloride solutions covering the same range of tonicity.

Measures causing the release of enzyme from insoluble material

Dilution with water. On dilution with water, enzyme was released from the particles in homogenates prepared in isotonic sucrose or sodium chloride solution, to give a distribution identical to that obtained by homogenizing directly in media of the same final tonicity. The change in the distribution of the enzyme appeared to be instantaneous.

Incubation at 37°. It was previously found for water homogenates (Kerr & Levvy, 1951; Walker & Levvy, 1951) that incubation in acetate buffer, pH 5·2, at 37° for 4 hr., converted all the enzyme present into a soluble form, with little change in the microscopic appearance of the insoluble particles. This property of acetate was not shared by citrate, nor did release of enzyme from the particles occur on incubating unbuffered preparations.

Homogenates in 0·17 M-sodium chloride or 0·25 M-sucrose solution behaved like water homogenates in that incubation for 4 hr. after buffering to pH 5·2 with acetate (final concentration 0·1 N) without change in tonicity resulted in the conversion of enzyme into a soluble form, although conversion was not so complete as in water. When acetate was replaced by citrate buffer (final concentration 0·1 N)

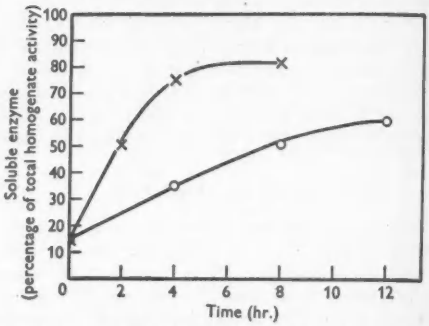

Fig. 3. Changes in the partition of the glucuronidase activity in a homogenate of adult mouse liver in 0·17 M-NaCl solution after incubating at 37° for varying periods. Acetate-soluble enzyme expressed as a percentage of the total homogenate activity. ○—○, unbuffered homogenate; ×—×, homogenate made 0·1 N with respect to acetate buffer, pH 5·2.

at pH 5·2, or when the unbuffered preparation was incubated at its natural pH (6·5–6·8), the release of enzyme from the particles was much slower and tended to a maximum at the figure for soluble enzyme in a water homogenate. This is illustrated for a saline homogenate in Fig. 3. In the unbuffered preparations incubation resulted in a change in the microscopic appearance of the particles, resembling that observed when homogenates in isotonic media were diluted with water (see 'Microscopic observations'). No enzyme inactivation was observed in these experiments.

Mechanical disintegration. As previously noted for water homogenates (Walker & Levvy, 1951), shaking with Ballotini in the Mickle tissue disintegrator caused almost complete release of the enzyme from the particles in a homogenate in isotonic sodium chloride or sucrose solution, without appreciable inactivation. The Waring Blendor has been found to act similarly in this respect (Table 3), practically all the enzyme in liver or a liver homogenate being converted to a soluble form in 3 min., without any loss in overall activity. At this stage, all the components of the preparation were seen under the microscope to have undergone fragmentation, with complete loss of structure.

It has been customary to use the supernatants from water homogenates for the assay of tissue glucuronidase activity (see Kerr & Levvy, 1951). It can be seen from Table 3 that the use of the Waring Blendor instead of the glass homogenizer can lead to a very different value for the glucuronidase activity of a tissue in such methods of assay (cf. Fishman & Talalay, 1947).

Table 3. *Fractionation of adult mouse-liver glucuronidase activity after treatment for varying periods in the Waring Blendor*

(The starting material consisted of liver which had previously been ground in water, 0·17M-NaCl or 0·25M-sucrose solution in the glass homogenizer for 1 min., or the whole organ to which water was added in the blender.)

Period of treatment (min.)	Acetate-soluble enzyme (% total activity)			
	Water homogenate	Saline homogenate	Sucrose homogenate	Liver and water
0	57*	14	14	—
0·5	71	77	72	—
1	88	89	86	79*
2	95	96	93	90
3	100	100	98	98
5	100	100	100	100

* These figures give a direct comparison of liver treated with water for 1 min. in the glass homogenizer and the Waring Blendor respectively.

Freezing. As in water homogenates (Walker & Levvy, 1951), the particles in sucrose or saline homogenates of liver gave up their glucuronidase activity to the solution on repeated freezing with a mixture of solid carbon dioxide and acetone, and thawing at 37°. The rates of transfer were, however, very different in the two types of isotonic homogenate. In sodium chloride solution, 60% of the enzyme activity became soluble after a single freezing, whereas four freezings were necessary before this stage was reached in sucrose solution.

Treatment with acetone. After homogenizing mouse liver in acetone (Walker & Levvy, 1951) followed by resuspension of the dry powder in water, 0·17M-sodium chloride or 0·25M-sucrose solution, almost all the enzyme activity was soluble.

Treatment with surface-active agents. The actions of Teepol XL (British Drug Houses Ltd), Triton X-100 and Triton A-20 (Rohm and Haas Co.) on homogenates in isotonic media were identical in all respects with their actions on water homogenates (Walker & Levvy, 1951). The first two agents in suitable concentrations caused complete conversion of the enzyme into a soluble form, but Teepol XL differed from Triton X-100 in that high concentrations were inhibitory. Triton A-20 had no effect of any kind.

Distribution of the enzyme according to particle size

Fractional sedimentation of mouse-liver homogenates in 0·25M-sucrose solution was carried out by a procedure which followed that of Schneider & Hogeboom (1950) as closely as our equipment permitted.

Fraction 1. A 10% homogenate (10 ml.) was centrifuged for 10 min. at 700 *g* and the supernatant was decanted. The residue was resuspended in 8 ml. isotonic sucrose solution and sedimented as before. The debris, consisting of nuclei contaminated with erythrocytes, unbroken liver cells and cytoplasmic granules, was resuspended for assay.

Fraction 2. The combined supernatants from fraction 1 were centrifuged at 5000 *g* for 10 min., and the supernatant was decanted and again centrifuged at 5000 *g* for 10 min. Two washings of the combined sediments were carried out by suspending the solid in 5 ml. sucrose solution and centrifuging at 20 000 *g* for 10 min. The debris, a fairly homogeneous preparation of the larger cytoplasmic granules, was finally resuspended for assay.

Fraction 3. Values for this fraction, consisting of all remaining insoluble material in the preparation, were found by difference, since centrifuging at 25 000 *g* (the maximum R.C.F. value at our disposal) did not give a sufficiently well packed sediment for clean separation. The supernatant and washings from fraction 2 were combined and aliquots were taken for assay. The figures thus obtained and those for fraction 4 gave by difference the figures for fraction 3.

Fraction 4. The remainder of the preparation was centrifuged at 25 000 *g* for 20 min. and the supernatant was decanted. After recentrifuging at the same speed, samples of the now optically clear supernatant were withdrawn directly from the centrifuge tube for assay of the 'soluble enzyme' in the homogenate.

Results for representative experiments are shown in Table 4. The main points emerging from this table are as follows. The total enzyme activity of the homogenates was fully accounted for by the activities of the separated fractions, and 80–90% was sedimentable. Of this, four-fifths was in the pure granule fractions, which also showed the highest specific activities. The relatively low activity of the impure nuclear fraction may well have been due to the contaminating granules.

Infant liver and liver regenerating after partial hepatectomy showed a similar distribution of insoluble enzyme activity to that seen in the normal adult tissue. The higher total and specific activities of growing and regenerating liver (cf. Levvy *et al.* 1948) were reflected in every centrifuge fraction.

All fractions of the sedimentable material showed approximately equal (60–70%) release of enzyme

activity to the solution on dilution with water. Under the microscope, there was no perceptible fragmentation of the particles in fraction 2. In infant as compared with adult liver a greater percentage of the enzyme in every fraction (70–80%) was converted to the soluble form.

in a given preparation no matter which method of treatment was adopted, but the results make it clear that it was unnecessary to take more than part of the enzyme into solution before the preparation displayed its full potential activity. The release of enzyme activity from insoluble material was readily

Table 4. *Fractional sedimentation of unbuffered mouse-liver homogenates in* 0·25 M-*sucrose solution*

Fraction no. and description	N (total)		Enzyme activity		
	mg./g. liver	% total	G.U.*/g. liver	% total	G.U.*/mg. N
Normal adult liver					
Total homogenate	39·0	100	3580	100	92
1. Nuclei, etc.	7·5	19	510	14	68
2. Large granules	6·5	17	1100	31	170
3. Small granules	6·8	17	1430	40	210
4. Soluble enzyme	16·5	42	470	13	29
Sum of fractions	37·3	95	3510	98	—
Infant liver (4-day-old)					
Total homogenate	31·0	100	5100	100	164
1. Nuclei, etc.	6·5	21	750	15	117
2. Large granules	5·1	16	1310	26	256
3. Small granules	5·3	17	2000	39	377
4. Soluble enzyme	14·2	46	950	19	67
Sum of fractions	31·1	100	5010	99	—
Regenerating liver (4 days after partial hepatectomy)					
Total homogenate	36·6	100	4480	100	122
1. Nuclei, etc.	7·2	20	550	12	76
2. Large granules	7·0	19	1360	30	194
3. Small granules	5·9	16	1820	41	309
4. Soluble enzyme	17·0	46	820	18	48
Sum of fractions	37·1	101	4550	101	—

* For definition of G.U. see text.

The activity of the enzyme in homogenates in isotonic media

Kerr & Levvy (1951) found that the use of too high a tissue concentration in the assay of water homogenates of mouse liver led to false low results for the total glucuronidase activity. Full activity was shown at all concentrations once the enzyme had been completely converted into a soluble form by incubation in acetate buffer at 37° for 4 hr.

Table 5 shows that tissue concentration had a similar effect in the assay of homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The activity no longer varied with tissue concentration once the enzyme had been completely converted into a soluble form. Even at low concentrations, however, homogenates in isotonic media, unlike those in water, increased considerably in activity when the enzyme was made soluble.

Table 6 shows parallel figures for the total glucuronidase activity and the percentage soluble enzyme activity in homogenates of adult mouse liver after treatment by various methods which cause the transfer of enzyme activity from insoluble material to the solution. The same final activity was reached

controlled when it was brought about by dilution with water or incubation in acetate buffer, and with these measures full activity was reached when only about half the enzyme had gone into solution, i.e. when the distribution of enzyme activity had become similar to that seen in water homogenates. Whereas these measures released the enzyme by a gradual process affecting all insoluble particles equally, mechanical disintegration in the tissue disintegrator or the Waring Blendor resulted in complete destruction of individual particles and thus did not produce complete activation in the present experiments until nearly all the enzyme had been brought into solution. The release of enzyme activity from insoluble material by the surface-active agents Triton X-100 and Teepol XL was difficult to control in degree, but the figures given in Table 6 show that when this process was complete the enzyme was fully active.

Since in the assay the incubation with substrate was carried out in acetate buffer, in which activation of the enzyme must have occurred, the figures given in Table 6 for the total glucuronidase activity of homogenates are subject to error. This consideration does not apply to the figures for soluble enzyme

activity, this fraction of the enzyme being already fully active.

The error arising from the activation of the enzyme during the incubation with substrate is illustrated in Fig. 4 by velocity curves for the hydrolysis of phenolphthalein glucuronide in acetate buffer by mouse liver homogenized in 0·17M-sodium chloride. When the homogenate (2·5%, w/v) was added to the usual buffer-substrate mixture, the rate of hydrolysis gradually increased until, after about 45 min., it reached the value obtained with a fully activated sample of the same homogenate. When the buffer-substrate mixture was made 0·17 M with respect to sodium chloride, so as to prevent dilution of the homogenizing medium in the enzyme preparation, the change in enzyme activity was slower, and full activity was only reached after 90 min. incubation. This concentration of sodium chloride did not inhibit fully activated preparations of the enzyme.

Similar results were obtained in experiments with homogenates in isotonic sucrose solution, except in that activation proceeded more slowly than in sodium chloride solution. Sucrose in a final concentration of 0·25 M was found to cause about 10% inhibition of fully activated glucuronidase preparations.

Replacing acetate by citrate buffer during the incubation at pH 5·2 not only slowed down the transfer of enzyme activity from insoluble material to the solution (see above), but also decreased the rate of activation of homogenates in isotonic media.

Table 5. *Variations in the glucuronidase activity of adult mouse liver with the homogenate concentration taken for assay*

(All results refer to a single sample of tissue; activities were measured in acetate buffer before and after conversion of all the enzyme into a soluble form by treatment for 20 min. in the tissue disintegrator.)

Concentration of homogenate* (g. tissue/100 ml. homogenizing fluid)	Glucuronidase units/g. liver			
	Homogenate in 0·17 M-NaCl		Homogenate in 0·25 M-sucrose	
	Before treatment	After treatment	Before treatment	After treatment
40	1320	3200	1200	3240
20	1680	3200	1560	3200
10	2200	3200	2120	3240
5·0	2400	3280	2240	3200
2·5	2380	3240	2200	3240
1·0	2300	3200	2100	3240

* 0·5 ml. of homogenate of the concentration shown was incubated with 0·5 ml. substrate solution and 3 ml. buffer in the usual way.

Table 6. *Effects of various measures on the total and soluble glucuronidase activity in homogenates (2·5%, w/v) of mouse liver in isotonic* NaCl *or sucrose solution*

(Activities measured in acetate buffer, and results expressed as percentages of the activity of the whole homogenate after 10 min. treatment in the tissue disintegrator.)

Treatment		Homogenate in 0·17 M-NaCl		Homogenate in 0·25 M-sucrose	
Agent	Extent	Total activity	Soluble activity	Total activity	Soluble activity
None	—	75	12	66	8
Tissue disintegrator	1 min.	82	50	79	55
	2 min.	91	72	88	78
	5 min.	100	98	100	97
	10 min.	100	100	100	100
Waring Blendor	0·5 min.	88	68	87	66
	1 min.	94	85	95	83
	3 min.	100	100	101	100
	5 min.	99	99	101	103
Triton X-100	0·1%	101	101	102	102
Teepol XL	0·4%	101	98	101	97
Water	½ vol.	82	14	75	17
	1 vol.	90	23	86	29
	3 vol.	100	47	97	51
Incubation in acetate buffer, pH 5·2	1 hr.	87	26	83	18
	2 hr.	92	41	91	30
	4 hr	100	80	100	69

The rate of activation in citrate buffer became even slower when 0·17M-sodium chloride was present, and slower still when this was replaced by 0·25M-sucrose. Velocity curves for the hydrolysis of phenolphthalein glucuronide in sucrose-citrate (pH 5·2) by homogenates of adult and infant mouse liver in 0·25M-sucrose solution are shown in Fig. 5.

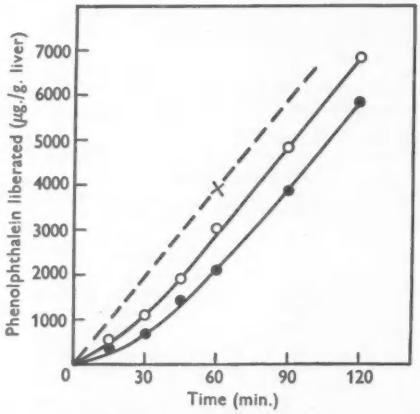

Fig. 4. Rate of hydrolysis of phenolphthalein glucuronide (0·00125M) in acetate buffer (final concentration 0·075N, pH 5·2) by a homogenate of adult mouse liver in 0·17M-NaCl solution. Final tissue concentration 0·3% (w/v). ●—●, buffer and substrate made 0·17M with respect to NaCl; ○—○, no NaCl added to buffer or substrate; – – × – –, assay of homogenate after treatment for 10 min. in the tissue disintegrator.

This graph illustrates the difficulties which would attend attempts to make comparative measurements of mouse-liver glucuronidase activity in homogenates in isotonic media without preliminary activation. It can be seen, however, that in untreated preparations the activity of the infant tissue was at all stages higher in terms of μg. phenolphthalein liberated/g. liver than that of the adult tissue (cf. Levvy *et al.* 1948).

The nature of the inactive enzyme

Table 7 shows the effect of varying the substrate concentration on the activity, expressed as glucuronidase units, of different preparations of the same tissue added to incubation mixtures of varying composition. When a homogenate in 0·17M-sodium chloride solution was incubated with increasing concentrations of phenolphthalein glucuronide in acetate buffer, inhibition of glucuronidase activity by excess substrate was seen, whether or not the enzyme preparation had been subjected to prior activation. Considerable activation of the untreated preparation must of course have occurred during the assay in acetate buffer. When a homo-

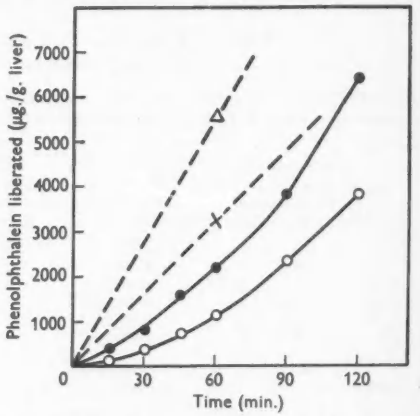

Fig. 5. Rate of hydrolysis of phenolphthalein glucuronide (0·00125M) in citrate buffer (final concentration 0·075N) by homogenates of adult and infant mouse liver in 0·25M-sucrose solution. Final tissue concentration 0·3% (w/v). Substrate and buffer made 0·25M with respect to sucrose. ○—○, adult; ●—●, infant (4-days-old); – – × – –, assay of adult liver after treatment of homogenate for 10 min. in the tissue disintegrator. – –△– –, assay of infant liver after treatment of homogenate for 10 min. in the tissue disintegrator.

Table 7. *Effect of varying substrate concentration on the rate of hydrolysis of phenolphthalein glucuronide in* 0·1N-*acetate or citrate buffer by homogenates of adult mouse liver in* 0·17M-NaCl *or* 0·25M-*sucrose solution*

(All results refer to a single sample of tissue; G.U./g. liver measured at pH 5·2 before and after treatment for 10 min. in the tissue disintegrator.)

Substrate concentration (M)	Saline homogenate in acetate buffer		Sucrose homogenate in citrate buffer		Sucrose homogenate in sucrose-citrate buffer	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
0·00125	2640	3600	2000	3600	1320	3200
0·00438	2320	3120	1960	3040	1440	2640
0·00875	2000	2480	2000	2400	2080	2240

genate in 0·25 M-sucrose solution was added to citrate buffer containing the same concentration of sucrose and a varying concentration of substrate, increase in the latter caused a rise in the net activity of the untreated homogenate, but a fall in the activity of the activated preparation. Whatever the factor may be that is responsible for the low activity of glucuronidase in mouse liver homogenized in isotonic media, it can thus to some extent be overcome by the use of a substrate concentration in itself high enough to inhibit the enzyme. These two opposing effects of increasing the substrate concentration on the net activity of an untreated preparation were exactly balanced when a sucrose homogenate was incubated with a mixture of citrate buffer and phenolphthalein glucuronide made up in water alone.

The results shown in Table 7 are entirely explicable on the view that the enzyme in a homogenate of mouse liver in isotonic media, but not in water, is subject to the action of an endogenous competitive inhibitor. An alternative explanation must, however, be considered, namely that the enzyme associated with the insoluble particles is surrounded by material which the substrate only penetrates with difficulty.

DISCUSSION

Accepting that the localization of an enzyme in the intact cell can be inferred from its distribution amongst the components of an isotonic homogenate, it is concluded from the present study that all or almost all of the glucuronidase activity of mouse liver is associated *in vivo* with subcellular particles, especially the large and small cytoplasmic granules.

The enzyme can be irreversibly released from the particles by various treatments which produce either swelling or fragmentation of the particles. The small amount of the enzyme which is present in a soluble form in isotonic preparations may be derived from the particles when they are liberated into the medium or it may arise from damage to the particles in the homogenizer. The amount of this soluble fraction is, however, not reduced either by employing hypertonic media which preserve better the intracellular appearance of the particles (Hogeboom *et al.* 1948) or by a very gentle grinding of the tissue. As the medium is made increasingly hypotonic there is a transfer of enzyme activity from the particles to the solution till, at low sucrose or saline concentrations, the distribution characteristic of a water homogenate is approached. It may be significant that the release of enzyme activity which occurs on prolonged incubation of a homogenate in an isotonic medium also ceases when the soluble activity reaches the figure for a water homogenate.

The distribution of glucuronidase in infant and regenerating liver is similar to that in normal adult liver, and the higher enzyme activity of these tissues is spread over particles of all sizes. In actively proliferating as compared with resting tissue, a greater proportion of the enzyme in all fractions of the sedimentable material is released in water. This agrees with the observation of Kerr & Levvy (1951) who found that in water homogenates the increase in the soluble activity accounted entirely for the higher overall activity of proliferating tissues.

As enzyme is released from the particles in homogenates in isotonic media, there is an increase in the activity of the enzyme remaining bound to insoluble material. Two explanations have been suggested for the initially low glucuronidase activity of these homogenates, namely the existence of a competitive inhibitor in the homogenate, or of material which hinders access of the substrate to the enzyme in the insoluble particles. The latter explanation is considered to be the more satisfactory since it covers the behaviour of water homogenates. In hypotonic media, a change in the particle occurs, permitting the escape of some of the enzyme from the particles. This change presumably permits much easier access of the substrate to the enzyme remaining within the particles. The existence of a competitive inhibitor is hard to imagine since it would have to be very selective in its action, affecting the insoluble enzyme in homogenates in isotonic media, but neither the enzyme in completely soluble preparations, nor the insoluble enzyme fraction in water homogenates.

Various factors have been found to influence results obtained in the assay of glucuronidase associated with insoluble material. It is clear that reliable comparative measurements of tissue activity can only be made after complete solution or complete activation of the enzyme. The physiological significance of results thus obtained is, however, another matter. If one accepts the premise that the state of an enzyme in homogenates in isotonic media approximates to its state in the intact tissue (Schneider & Hogeboom, 1951), the possibility must be considered that factors such as those encountered in the present work have effects on the activity of the enzyme in living tissues, which may be as important as changes in the active protein content, detected by the use of soluble preparations.

SUMMARY

1. The partition of glucuronidase activity between the subcellular particles and the solution has been studied in homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The particles were sedimented by centrifuging at a high speed, or by centrifuging at a low speed after agglutination with acetate buffer.

2. The two methods of sedimentation gave identical results and 80–95 % of the enzyme activity was found in the precipitate.

3. As the medium was made increasingly hypotonic, a greater proportion of the enzyme activity was released from the particles. Particles of different sizes, separated by fractional sedimentation, behaved comparably in this respect.

4. The distribution of enzyme activity over particles of different sizes was the same in infant liver and in liver regenerating after partial hepatectomy as in the normal adult tissue, but preparations of the growing or regenerating tissue showed greater release of enzyme activity to the solution on dilution with water.

5. The homogenates in isotonic media did not display their full glucuronidase activity on direct assay, but partial activation occurred under assay conditions. Full activity was displayed when about half the enzyme present was brought into solution.

6. The possible mechanism of activation and its physiological significance are discussed.

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The Chemical Composition and Structure of the Yeast Cell Wall

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The chemical composition of the yeast cell wall was first studied by Salkowski (1894), who investigated the polysaccharide material which remained after intact cells had been digested with dilute sodium hydroxide solution. Zechmeister & Tóth (1934) continued the study, but again disrupted the cells by fairly vigorous chemical action. They suggested, however, that an enzymic method might do less damage to the cell wall, and indeed later they isolated the glucan component of the cell wall by the action of pepsin and amylase on an autolysed yeast suspension (Zechmeister & Tóth, 1936). These chemical and enzymic investigations have indicated that several polysaccharides may be present in the cell wall, and it has been suggested that, besides the glucan, a mannan (Haworth, Hirst & Isherwood, 1937) and possibly a 'glycogen' (Ling, Nanji & Panton, 1925) may form part of the structure; no direct evidence for this has been given. The structure of these polysaccharides has been investigated by various workers. The general method of isolation has been extraction of whole yeast with 3 % sodium hydroxide solution at 100°, whereby the mannan and some glycogen go into solution, whereas the glucan and most of the glycogen remain as the insoluble material, which is seen under the microscope to retain the shape of the cell, and is therefore assumed to be part of the cell wall. The glucan was shown to contain only glucose with β-1:3 linkages between the radicals, by Zechmeister & Tóth (1934) and Hassid, Joslyn & McCready (1941); more recently Bell & Northcote (1950) found that this polysaccharide was highly branched and determined its average chain length. The structure of the mannan has been studied by Haworth *et al.* (1937), Haworth, Heath & Peat (1941) and Lindstedt (1945), but the constitution of the glycogen, and especially its possible existence in two forms (Ling *et al.* 1925; Daoud & Ling, 1931) has received little attention.

In the present work the yeast cell wall has been isolated in two ways:

A. By physical methods entailing mechanical breakage of the cell and isolation of the washed cell wall by differential centrifugation.

B. By chemical methods, entailing breakage and isolation, similar to those used by the previous workers, i.e. digestion of the whole cell with 3% sodium hydroxide.

In this way a more complete and quantitative survey of the substances making up the structural elements of the outer cell wall has been obtained than was hitherto possible. Both a chemical and a microscopical examination of the preparation have been made and in this latter connexion the material has been found to be very suitable for investigation by the electron microscope.

EXPERIMENTAL

Material used and general analytical methods

The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers' Co. Ltd.). Its dry weight, determined under the same conditions as those used with the cell wall preparations, was 27·3% of the moist weight.

All the analyses were carried out on material dried at 0·01 mm. Hg over P_2O_5 at room temperature.

Total N (Kjeldahl) was determined using Nessler's reagent (Umbreit, Burris & Stauffer, 1949). Total P was determined according to Fiske & Subbarow (1925, cf. Umbreit *et al.* 1949).

Chromatography of sugars. Descending chromatograms were run on Whatman no. 1 papers with *n*-butanol/water at 37° for 60 hr. (Hough, Jones & Wadman, 1950). Glucose, galactose, glucosamine, mannose and arabinose were used as markers. The spots were coloured with aniline hydrogen phthalate (Partridge, 1949) and ammoniacal $AgNO_3$ on duplicate papers.

Chomatography of amino-acids. Descending chromatograms were run with phenol/0·3% (w/v) aqueous NH_3 at 20° in an atmosphere of coal gas (Consden, Gordon & Martin, 1944). The spots were coloured with 0·3% (w/v) triketohydrindene hydrate in *n*-butanol.

The solutions applied to the chromatograms were adjusted so that the approximate concentration of the sugars and the amino-acids was never less than 1%.

The work below is described in two parts, dealing with cell wall material obtained by mechanical breakage of the cell, preparation *A*, and that obtained by chemical cytolysis of the cell, preparation *B*.

Preparation *A*

Isolation of the cell wall material by mechanical breakage of the cell

Yeast (700 mg.) was suspended in 10 ml. of water with 4 g. of fine glass beads (Ballotini no. 12, Chance Bros Ltd., Birmingham) and the mixture placed in a vertical cup (internal measurements 5 × 2·2 cm.) of a Mickle cell disintegrator (Mickle, 1948). Vibration was continued for 30 min., after which the resultant suspension was decanted away from the glass beads. The supernatant was centrifuged at 1500 *g* for 10 min. (height of tube 10 cm.), and the residue washed repeatedly with water until the final supernatant became clear; the residue comprised the required cell wall fraction. The initial supernatant and the washings were combined and centrifuged at 14000 *g* for 20 min. (height of tube 10 cm.), when a residue of fine particles was obtained. The final supernatant and the two residues were freeze-dried. The yields of material from three representative fractionations are shown in Table 1. The beads cannot have contributed much to these fractions since their ash content never exceeded 3%.

Table 1. *Yield of fractions obtained by differential centrifugation of mechanically disintegrated yeast cells*

	mg./100 mg. dry wt. taken			
Exp. no.	Residue deposited at 1500 *g* (cell wall fraction)	Residue deposited at 14000 *g* (small particle fraction)	Supernatant ('soluble' material)	Recovery (%)
1	15·3	30·1	44·4	89·8
2	14·1	28·4	41·8	84·3
3	14·5	30·3	42·0	86·8

Microscopical examination of cell wall

The cell walls isolated in the above manner are Gram-negative, whereas the whole yeast cells are Gram-positive. It is thus an easy matter to distinguish any unbroken cells in the preparations by a microscopical examination of stained films. Many fields of numerous preparations were examined and in no case was a whole cell detected. The cell walls were also examined by the phase-contrast microscope and here again no whole cells and very little debris could be detected. It is concluded that the material isolated does represent solely the outer membranes of the cell, and that chemical investigations carried out on it will in fact characterize the cell wall which is thought to be made up of these membranes.

Electron microscope. A comprehensive examination of the cell wall was made under the electron microscope using both Siemens and Radio Corporation of America (R.C.A.) instruments. The high tension voltages used ranged from 50 to 90 kV. The material was suspended in water and allowed to dry at room temperature in air on the filmed specimen grids. Films of two types were used as supporting membranes, these being mounted on standard Kodak copper grids. Early preparations were mounted on nitrocellulose supporting films, but owing to the relatively large size of yeast cells these films frequently ruptured during investigation in the microscope. 'Formvar' (polyvinyl formal) films prepared from dioxan solution were found to be much stronger, particularly when large numbers of cells were present in the field under examination. Preparations were shadowed with an alloy of Au and Pd employing the usual shadow-casting techniques. The shadowing angle was 45°.

The most characteristic and obvious detail in the structure of the cell walls was the occurrence of scars on the surface; these appeared on the majority of the larger cells but not on the smaller cells. Some cells carried as many as sixteen such markings (Fig. 4*a*). By comparisons with those cells to which were attached parts of the cell wall of the bud, and

with that of an intact cell in the process of budding (Fig. 1), these markings were identified as budding scars and appeared as circular thickenings of the cell surface (Figs. 2 and 4*a–d*). The figures are metal-shadowed electron micrographs. The bud scars could also be seen on the cell walls under the phase-contrast microscope. It is of interest to note that Dorsten, Oosterkamp & Le Poole (1947) used whole yeast cells for testing the 400 kV. experimental electron microscope and the pictures taken by their instrument show indications of these budding scars in the whole cell. When the preparations were treated with 2 N-HCl the scars appeared to be more resistant to chemical attack than the general cell surface. By observations of the edge of the cells and of the apparent peeling of the membranes which often occurred at the bud scar (Fig. 2) it could be seen that the cell envelope consisted of at least two membranes. When the preparations were extracted successively with methanol and ether (see below) to remove lipids, and re-examined, the division into two membranes became obvious (Fig. 5). However, after treatment with 3% (w/v) aqueous NaOH solution to remove mannan, protein, and lipid (see below) only one membrane is apparent at the bud scars (cf. Figs. 2 and 4).

The type of breakage resulting from disruption by glass beads, as applied to yeast cells, can be seen from the electron micrographs (Figs. 3 and 4*d*).

Chemical investigation of the cell wall

Elementary analysis. Total N, 2·1%, indicating a protein content of approx. 13%; total P, 0·31%.

Mineral content. When maintained at red heat in a platinum boat in a stream of clean air for 1 hr. 17·21 mg. yielded a white ash (0·55 mg., 3·21%).

Lipid content. The lipid was isolated by boiling the cell walls (17·39 mg.) in 95% (v/v) aqueous methanol (1 ml.) for 30 min. and subsequently extracting continuously with ether in a modified Soxhlet apparatus (Mitchell, 1951) at room temperature for 6 hr., the whole process being repeated three times. The ether was evaporated and the fat weighed directly. The yield was 1·48 mg. (8·5%). (Found: N, 0·1; P, 0·5%.) The fat thus appears to contain little phospholipid.

Acid hydrolysis. The preparation (8·40 mg.) was hydrolysed for 6 hr. with 1 ml. of 2 N-HCl in a sealed tube at 98°; it dissolved completely to give a very light brown solution. This was evaporated to dryness at 20° under reduced pressure and dissolved in 0·1 ml. of water. The resultant solution was investigated on paper chromatograms. Only two sugars were apparent in the hydrolysate, namely mannose and glucose; no glucosamine could be detected. Although hydrolysis of protein was probably incomplete, the hydrolysate also showed the presence of glutamic acid, aspartic acid, serine, glycine, threonine and alanine with only very faint indications of histidine, leucine and the other basic amino-acids.

Water content. The cell wall material (10·8 mg.) was dried over P_2O_5 at 100° and 0·01 mm. Hg for 8 hr.; there was no loss in weight. Dried material (100 mg.) exposed to air at room temperature for 48 hr. increased in weight by 11·2%.

Polysaccharides of the cell wall

Isolation and investigation of the glucan component. The cell wall material (97·21 mg.) was digested with 3% (w/v) aqueous NaOH (2·0 ml.) for 6 hr. at 100°. The supernatant obtained after centrifuging was retained for examination of the dissolved mannan (see below). The insoluble residue was extracted consecutively with 3% NaOH at 100° for 6 hr., 0·5 N-acetic acid (2 ml.) at 75° for 6 hr. and finally washed with ethanol (2 ml.) and ether (2 ml.). The white solid was dried over P_2O_5 at 0·1 mm. Hg. Yield 27·9 mg. (28·8% of dry matter taken). (Found: N, 0·3; P, 0·0%.)

Hydrolysis of 10 mg. of this substance with 2 N-HCl (1 ml.) in a sealed tube at 100° for 6 hr. gave a light-brown solution. This was freeze-dried and the resultant solid dissolved in 0·1 ml. of water. The chromatograms of this solution showed the presence of glucose only.

The isolated material contained 98% glucose when this was estimated by anthrone (Seifter, Dayston, Novic & Muntwyler, 1950). An equivalent amount of glucan isolated from whole yeast by the method of Bell & Northcote (1950) gave 97·4% glucose.

The substance isolated from the cell wall contained only glucose and was very insoluble in water, thus resembling the glucan isolated from whole yeast. It still retained in most cases the shape of the cell wall when examined under the microscope, and thus it constitutes at least part of a continuous membrane within the cell wall.

Isolation and investigation of the mannan component. The solution obtained in the above experiment was made just acid to methyl red with acetic acid, and the mannan was precipitated by 4 vol. of ethanol. The precipitate was centrifuged and separated from the supernatant, redissolved in water and reprecipitated. The white solid thus obtained was washed with ethanol and ether and dried in the normal manner. Yield, 30·1 mg. (31·0% of original dry matter). $[\alpha]_D^{20°}$ +87° (*l*, 2; *c*, 1 in water). (Found: N, 1·3; P, 0·26%.)

This material (20 mg.) was hydrolysed as above for the glucan and gave a colourless solution. The hydrolysate was freeze-dried and dissolved in 1 ml. of water. Mannose phenylhydrazone was prepared from this solution according to the method of Nowatnowna (1936). Yield 20·2 mg. (i.e. 60% mannose); m.p. 196° (uncorr.) not depressed on admixture of an authentic sample of mannose phenylhydrazone (m.p. 196°). The cell wall mannan (30·0 mg.) was further purified by precipitation and subsequent decomposition of the copper compound (Haworth *et al.* 1937). Yield 25·0 mg. (N, 1·0; P, 0·2%). $[\alpha]_D^{20°}$ +88° (*l*, 2; *c*, 1·0 in water). The hydrolysed mannan showed only mannose on a paper chromatogram. The mannan could also be extracted from the cell wall material by water at 100°, although the rate of extraction was considerably lower; 25 mg. gave 2·5 mg. of mannan after 6 hr. and a further 2·4 mg. after 12 hr. The mannan isolated in this manner had a relatively high nitrogen content (N, 2·5; P, 0·23%) and gave a positive biuret reaction.

Glycogen. No glycogen could be isolated from the cell wall preparation by means of 0·5 N-acetic acid (75° for 12 hr.) nor could any glycogen be detected in the cell wall by staining with iodine and subsequent microscopical examination. The small particle fraction isolated by differential centrifugation of the broken yeast cell did, however, stain a dark brown with iodine.

PREPARATION *B*

Isolation of the cell wall material by digestion of the cells with sodium hydroxide solution

The whole yeast (12·62 g.) was dispersed in 15 ml. of 3% (w/v) aqueous NaOH and heated on a boiling-water bath for 6 hr. The brown solution was centrifuged and the residue redigested with a further 15 ml. of 3% NaOH for 3 hr.; it was then washed with water, ethanol and ether and dried in the normal manner. Yield, 0·13 g.

Microscopical examination of cell wall

This cell wall material showed an electron-dense substance scattered in granular and particulate masses throughout the walls (Fig. 6). Part of this material seemed to be glycogen as it could be seen under the optical microscope to stain brown with iodine. It could be dissolved away with 0·5 N-acetic acid at 75° (see below). Apart from this the electron microscope examination of this preparation showed little difference from that of preparation *A*.

Chemical investigation of the cell wall

Elementary analysis. Total N, 0·7 %; total P, 0·07 %.

Acid hydrolysis. The hydrolysis was carried out in the same manner as for preparation *A*. Only glucose was apparent in the hydrolysate when this was examined on paper chromatograms.

Polysaccharides of the cell wall

Isolation and investigation of the glycogen component. The cell wall material (130 mg.) was digested with 0·5 N-acetic acid (5 ml.) for 6 hr. at 75°. The residue was centrifuged and re-extracted with acetic acid for a further 6 hr. The acetic acid solutions were combined and evaporated to small bulk under reduced pressure when the glycogen was precipitated from solution by the addition of 6 vol. of ethanol. Yield of glycogen 25·1 mg. (19·3 % of the cell wall).

The glycogen could not be extracted with water from the cell wall, but after removal by dissolving in the dilute acetic acid and precipitation by ethanol the resultant material was very soluble in water to give a clear solution. This solution gave a deep red-brown colour with iodine.

Isolation and investigation of the glucan component. The residue obtained in the above experiment during the isolation of the glycogen corresponded to the glucan. It was washed with water, ethanol and ether and dried in the usual way. Yield, 93·3 mg. (71 % of the cell wall). This glucan corresponded exactly in appearance, glucose content and solubility, with that isolated from whole yeast and from the cell wall preparation *A*.

DISCUSSION

The literature contains very little information concerning the chemical and enzymic nature of the outer cell walls of plant or animal cells, and it was with this in mind that we attempted in the present research to isolate a part at least of the cell wall of yeast which might lend itself to further studies. A preparation has been obtained by mechanical breakage and differential centrifugation which is not contaminated by whole cells or cell debris. This preparation can be made with a fairly constant composition, free from substances present in other fractions of the cell and thus a clear-cut separation of a definite morphological structure has probably been achieved. The wall as isolated is made up of two or more membranes; two in fact can be clearly seen by means of the electron microscope. It has been possible by relating the chemical investigation to the microscopical studies to show that one of these two apparent membranes is, in part, composed of the glucan polysaccharide since this polysaccharide can be isolated free from other material, still retaining the general shape of the whole cell and obviously constituting a complete membrane. The other membrane remains intact after removal of lipid; but when the protein and mannan are removed from the cell wall preparation by means of dilute sodium hydroxide solution it is no longer visible and thus this second membrane is made up in part of protein or mannan or both of these substances. The general analysis of the cell wall shows an approximate chemical composition of glucan 29%, mannan 31%, protein 13%, lipid (mainly neutral fat) 8·5 % and ash 3 % which accounts for over 84 % of the material. No other major constituent has been detected during the present investigation. Glycogen could only be obtained associated with the cell wall by using sodium hydroxide as a cytolysing agent. It is not present in the mechanically broken cell wall material, but appears among the fine particulate matter released by the breakage of the cell and the subsequent emptying of its contents. The glycogen present in the chemically prepared cell walls was seen to be present as scattered granules and it is thought that although it is probably closely associated with the cell wall in the living cell it does not form a structural part of the wall in the same way as the glucan and mannan.

The mannan can be extracted from the cell wall preparation *A* by means of hot water, and prepared in this manner it is found to be closely associated with protein which is extracted with it. This association in the cell wall had already been postulated by Garzuly-Janke (1940) from studies on the whole cell.

The observations carried out on the cell wall with the electron microscope and the phase-contrast microscope have shown clearly the existence on the cell surface of bud scars. These were first reported by Barton (1950) by microscopical examination of living and stained whole cells, although the observations were difficult and details of structure could not be obtained. The present work has shown that these scars are characterized by a circular thick edge slightly raised above the cell surface; the larger and presumably older cells carry a larger number of these scars. The edges of the scars are more resistant to chemical attack by dilute acid than is the general cell wall and this may indicate a difference in chemical composition. The existence of these scars must be important in relation to the process of budding and the behaviour of the cell wall as an osmotic boundary.

SUMMARY

1. A cell-wall fraction of yeast has been isolated after disintegrating the whole cells in a Mickle cell disintegrator and subsequently centrifuging.

2. The material isolated has been shown to be free of whole cells and cell debris.

3. A quantitative chemical analysis of the cell wall of yeast has shown it to be composed of protein, lipid and at least two polysaccharides, a mannan and a glucan. The glycogen associated with the cell wall preparation obtained by treatment of the whole cells with sodium hydroxide solution does not function as a structural element and is not present in the fraction obtained by mechanical breakage.

4. The mannan is associated with a protein present in the cell wall.

5. The cell wall has been examined by the optical and electron microscopes and has been shown to consist of at least two membranes, one of which is made up in part of the glucan component.

6. The existence of bud scars on the cell wall has been confirmed and some details of their structure observed.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Intact yeast cell showing budding.

Fig. 2. Group of cell walls (preparation *A*) showing bud scars and peeling of two apparent membranes at the scars.

Fig. 3. Cell wall (preparation *A*) showing junction between two budding cells together with other bud scars.

Fig. 5. Fragment of cell wall (preparation *A*) after extraction of lipid showing two distinct membranes.

Fig. 6. Cell wall prepared by cytolysing whole cells with 3% NaOH (preparation *B*) showing the granular appearance of the glycogen.

PLATE 2

Fig. 4. Cell wall (preparation *A*) treated with 3% NaOH. (*a*) Cell membrane showing numerous bud scars and disappearance of double membrane apparent in Fig. 2. (*b*), (*c*) and (*d*) Showing, in particular, details of structure of cell membrane at the budding point; note thickening and raised appearance of the membrane.

Fig. 1.

Fig. 2.

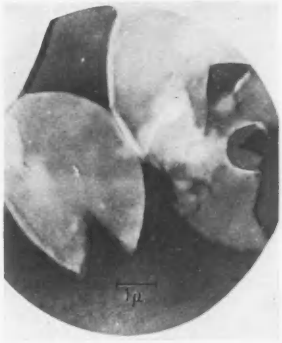

Fig. 3.

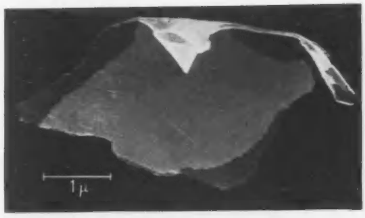

Fig. 5.

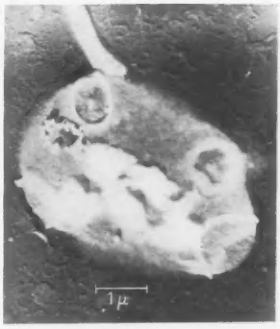

Fig. 6.

D. H. NORTHCOTE AND R. W. HORNE—THE CHEMICAL COMPOSITION AND STRUCTURE OF THE YEAST CELL WALL

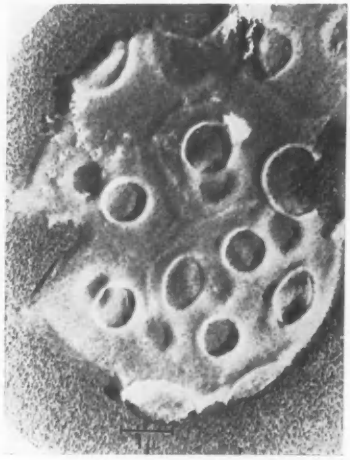

Fig. 4 *a*.

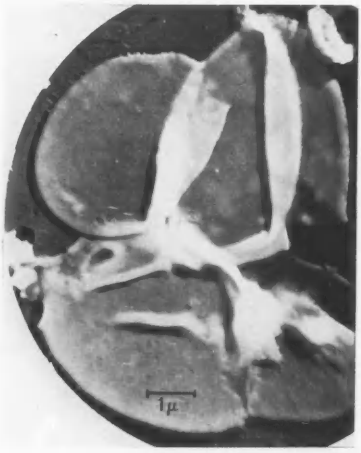

Fig. 4 *b*.

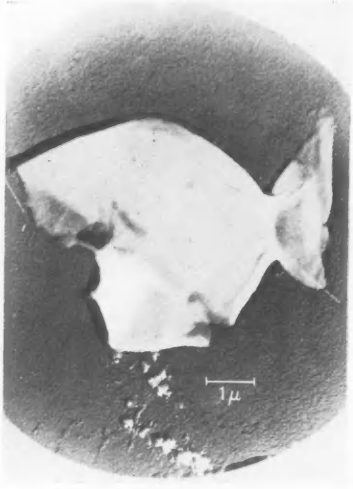

Fig. 4 *c*.

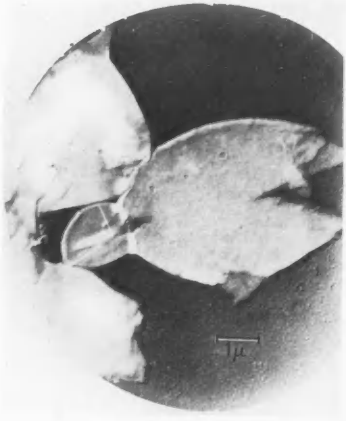

Fig. 4 *d*.

D. H. NORTHCOTE AND R. W. HORNE—THE CHEMICAL COMPOSITION AND STRUCTURE OF THE YEAST CELL WALL

The Estimation of Vitamin E

1. SEPARATION OF TOCOPHEROL MIXTURES OCCURRING IN NATURAL PRODUCTS BY PAPER CHROMATOGRAPHY

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(*Received* 30 *August* 1951)

The determination of the tocopherol content of biological materials by the ferric chloride-2:2′-dipyridyl method (Emmerie & Engel, 1938) gives results which are of limited value for two reasons. First, the biological activities of the α-, β-, γ- and δ-tocopherols are in the approximate ratio 100:30:20:1, depending on the biological test used, whereas all four tocopherols give the same value by the chemical method. Secondly, non-tocopherol substances which also give a red colour with the Emmerie-Engel reagents are often difficult to separate from the tocopherols and it is not always possible to be certain that the interfering impurities have been removed from the solutions which are finally tested (Baxter, Lehman, Hove, Quaife, Weisler & Stern, 1947).

Differential analysis of the tocopherols can be partly achieved by coupling the γ- and δ-compounds with diazotized *o*-dianisidine (Weisler, Robeson & Baxter, 1947); the α- and β-tocopherols do not couple with this reagent. Since β-tocopherol has been found in wheat products only, values for the total and γ-plus δ-tocopherol contents of most materials can be obtained by examining for (*a*) total tocopherols by the Emmerie-Engel method, and (*b*) γ-plus δ-tocopherols by coupling with diazotized *o*-dianisidine. The value for α-tocopherol can be obtained by subtracting (*b*) from (*a*).

The desirability of a method which would allow a clear-cut separation of the tocopherols led Emmerie (1949) to attempt the resolution of the tocopherols by chromatographing on Floridin *XS* Earth or alumina. Although the α- and γ-tocopherols could be separated under strictly controlled conditions, mixtures of the α- and β-tocopherols could not be resolved into the separate components.

Since paper chromatography has found application in numerous problems involving the separation of closely related compounds, attempts have been made in the present work to extend the method to the analysis of tocopherol mixtures made up in the laboratory. Tocopherol mixtures occurring in natural products have also been examined in order to test the utility of the method in distinguishing non-tocopherol reducing substances from authentic tocopherols.

EXPERIMENTAL

Chromatography

Both ascending (Williams & Kirby, 1948) and descending (Consden, Gordon & Martin, 1944) methods were used. Whatman no. 1 filter paper was coated with Vaseline (Winteringham, Harrison & Bridges, 1950) by dipping it in a 2·5% (w/v) solution of Vaseline in ether and then allowing the solvent to evaporate from it in the air. Storage of the coated papers for as long as 2 months before use did not alter their properties. The tocopherols were applied to the papers by means of a calibrated pipette delivering 6·3 μl. Alcoholic solutions of the tocopherols were used to avoid dissolving the Vaseline at the points of application. Various concentrations of methanol, ethanol and acetone in water were each tested for use as the mobile phase and 75% ethanol (v/v) was finally selected as giving the best separations.

Detection of the tocopherols

The developed chromatograms were dried for 15 min. at room temperature and then sprayed with 2:2′-dipyridyl (0·25%, w/v) in ethanol followed by ferric chloride (0·1%, w/v) in ethanol. Bright-red spots were formed on a white background. The background gradually assumes a pink colour so the positions of the spots must be marked fairly soon after spraying. In order to obtain a permanent record, methanolic silver nitrate (1·7 g. $AgNO_3$ in 50 ml. water, 33 ml. methanol and 17 ml. aqueous NH_3 sp.gr. 0·880) can be used. The dark brown spots appear about 1 min. after spraying, the one corresponding to the α-form appearing first and that corresponding to the δ-form last. Spraying with 2% (w/v) Na_2CO_3 followed by diazotized *o*-dianisidine solution (prepared according to the method of Weisler *et al.* 1947) was used for detecting the γ- and δ-tocopherols. The γ-compound gives a dark-purple spot, whereas the δ-compound gives a red spot readily distinguishable from the γ-spot. Under these conditions the α- and β-compounds do not give spots.

Separation of synthetic mixtures of the tocopherols

Mixtures of racemic α-, β- and δ-tocopherols were readily separated using 75% (v/v) aqueous ethanol as the mobile phase and Vaseline-coated filter papers as the stationary phase; the β- and γ-isomers could not be separated. The R_F values of the tocopherols were: α, 0·50; β, 0·72; γ, 0·72; δ, 0·84. These values were the same whether the tocopherols were used alone or mixed with other tocopherols.

Inability to separate the β- and γ-isomers is not a serious

drawback in examining natural products because the β-compound occurs only in wheat products. In addition, if a spot is obtained at $R_F = 0{\cdot}72$ using the 2:2′-dipyridyl-ferric chloride reagents, the presence of γ can be ascertained by using the *o*-dianisidine spray.

As little as 3 μg. of any of the tocopherols can be readily detected with the dipyridyl-ferric chloride reagents and even less of the γ- and δ-compounds can be detected with the sodium carbonate-*o*-dianisidine spray.

Extraction of the tocopherols from biological material

Methods for the extraction of tocopherols from natural products are largely based on methods for fat extraction (Quaife & Harris, 1948; Quaife & Dju, 1949). Lipids and sterols interfered with the running of the tocopherols on the chromatograms and were consequently removed. The lipids were hydrolysed in the presence of pyrogallol (Tošić & Moore, 1945) to prevent loss of tocopherols by oxidation, and the unsaponifiable matter freed from sterols by crystallizing from methanol solution at −15°. Carotene in large amounts also caused interference on the chromatograms; consequently the carotenoids were removed by absorbing them from benzene solution on Floridin Earth according to the method of Emmerie & Engel (1939). The Floridin Earth 'specially prepared for use in Emmerie's test for tocopherols' as supplied by British Drug Houses Ltd. was found to cause some destruction of the tocopherols. Glavind, Kjølhede & Prange (1942) have also reported that in the passage of tocopherols through earths of high surface activity a considerable amount of the tocopherols is oxidized. They prevented the oxidation by boiling the earth (1·5–2·0 g.) with $SnCl_2$ (0·25 g.) and 10 N-HCl (5–8 ml.) before using it. This procedure has been adopted in the present work, the heated mixture being introduced into an adsorption tube and washed free from acid with ethanol (5 ml.) followed by benzene (5 × 5 ml. portions). Using this method of preparation quantitative recoveries of the tocopherols have been obtained using as little as 10 μg. of tocopherols. Some, if not all, of the destruction of the tocopherols when using B.D.H. Floridin Earth was due to the presence of iron in the earth.

Examination of tocopherol mixtures present in natural oils

Vegetable oils are among the richest natural sources of the tocopherols, and consequently afford excellent starting materials for testing the method described. The oils were hydrolysed (Tošić & Moore, 1945) and the unsaponifiable fraction in benzene passed through a prepared column (30 × 13 mm.) of Floridin Earth (see above). For each oil the filtrate was evaporated under reduced pressure and the residue dissolved in warm methanol. After removing the sterols by crystallizing at −15°, the mother liquors were applied to the starting line of the paper. The tocopherols found in several oils are shown in Table 1.

Treatment of the chromatogram of the wheat-germ oil with the 2:2′-dipyridyl-ferric chloride reagents gave, in addition to spots at the positions corresponding to the α- and β-tocopherols, a distinct spot at $R_F = 0{\cdot}93$. Ammoniacal silver nitrate was reduced by the substance, but a spot was not obtained with the *o*-dianisidine reagent. The identity of this substance was not ascertained. The spot at $R_F = 0{\cdot}72$ was β- and not γ-tocopherol because spraying with sodium carbonate and diazotized *o*-dianisidine failed to give a spot. None of the other oils examined gave an 'apparent tocopherol' spot in this way, indicating that all the interfering substances had been removed prior to chromatography.

Table 1. *Tocopherols found to be present in various natural products*

Substance	Tocopherol α	β	γ	δ
Arachis oil	+	.	+	.
Cottonseed oil	+	.	+	.
Soya-bean oil	+	.	+	+
Wheat-germ oil	+	+	.	.
Cocksfoot grass	+	.	.	.
Fescue grass	+	.	.	.
Timothy grass	+	.	.	.
Cows' blood (cows at grass)	+	.	.	.
Cows' milk (cows at grass)	+	.	.	.

In the soya-bean oil chromatogram three spots with R_F values 0·50, 0·72 and 0·84 were obtained with the Emmerie-Engel reagents. The presence of γ- and δ-tocopherols was confirmed by means of the *o*-dianisidine spray.

Tocopherols occurring in other natural products

The importance of the tocopherols in the nutrition of farm animals is becoming increasingly apparent. Consequently, three different species of grass, timothy (*Phleum pratense*), cocksfoot (*Dactylis glomerata*) and fescue (*Festuca pratensis*), have been examined to determine which tocopherols they contain. The grasses were extracted with ethanol in a Soxhlet apparatus and the ethanol solution extracted with light petroleum (Quaife & Harris, 1948). The unsaponifiable fractions were examined in the same way as those from the oils. Only α-tocopherol was found in the grasses (Table 1) but there is present in cocksfoot a substance which gives a purple colour with Na_2CO_3 and diazotized *o*-dianisidine. This unidentified substance moved only a short distance from the starting line on the chromatogram (R_F approx. 0·05).

The blood and milk of cows feeding on the three grasses contained α-tocopherol only. Because of the relatively low levels of tocopherols in cows' blood and milk (about 800 and 150 μg./100 ml. respectively), even when the dietary intake is 10 g. or more daily (Cabell & Ellis, 1942), about 10 ml. of blood serum and 50 ml. of milk are necessary for a satisfactory analysis to be made.

DISCUSSION

The Emmerie-Engel method for determining tocopherols depends on the reduction of alcoholic ferric chloride followed by reaction of the ferrous salt with 2:2′-dipyridyl to give a red ferrous-dipyridyl complex. Since other substances likely to be present in the fat-soluble fraction of natural products can also reduce ferric to ferrous iron, these interfering substances must be removed in order to obtain the true tocopherol content. Even then comparison with biological assays is difficult because of the different biological potencies of the tocopherols, and the fact that the Emmerie-Engel reaction does not distinguish between the different compounds.

The method described in the present work allows the purity of the solution under test for tocopherols to be assessed and also enables the separation of the commonly occurring α-, γ- and δ-tocopherols to be achieved. Applications of the method to the examination of several oils indicate the usefulness of the method. For example, the presence of δ-tocopherol in soya-bean oil is readily ascertained, whereas its existence escaped detection by conventional methods for many years. In addition, the substance in cocksfoot grass which gives a colour with diazotized *o*-dianisidine is shown to be different from any of the known tocopherols in its behaviour on the chromatogram. Similarly, a non-tocopherol ferric-reducing substance which is not removed by the saponification, adsorption and crystallization processes is present in wheat-germ oil.

Although the method will probably not be readily applicable to routine analysis, its value for reliably detecting the presence of naturally occurring tocopherols is apparent. Application of the method to the quantitative analysis of tocopherol mixtures is being undertaken and will be the subject of a further communication.

SUMMARY

1. A method is described for the detection of α-, β-, γ- and δ-tocopherols by filter-paper chromatography.

2. The method has been used for study of the tocopherols in various vegetable oils and grasses and also in blood and milk.

The author is grateful to Dr J. Green, Vitamins Ltd., for discussions on the use of Floridin Earth, and to the following for generous gifts of material: Prof. P. D. Boyer, Dr H. Dam, Dr A. Emmerie and Dr M. L. Quaife, Messrs J. Bibby and Son Ltd., Roche Products Ltd. and Vitamins Ltd.

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Flavin-adenine Dinucleotide and Diaphorase in Resting and Germinated Spores, and Vegetative Cells of *Bacillus subtilis* and *Bacillus megatherium*

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(*Received* 22 *May* 1951)

There are conflicting reports on the respiration rate of resting bacterial spores, but it is generally agreed that it is lower and less sensitive to cyanide than that of vegetative cells (Tarr, 1933; Keilin & Hartree, 1947). Keilin & Hartree (1949) have also found that although the spores of *Bacillus subtilis* National Collection of Type Cultures 85(N.C.T.C.) contain considerable amounts of unidentified haematin compounds, their cytochrome content is only 6% of that of vegetative cells, and more recently Chaix & Roncoli (1950) have observed development from an atypical to a classical cytochrome spectrum during the growth of several strains of *B. subtilis*. These findings suggest that bacterial spores differ from vegetative cells in possessing an alternative to the cytochrome-cytochrome oxidase system, possibly in the form of a flavoprotein reacting with oxygen, either directly or through an unidentified haematin catalyst (Slater, 1949*a*, *b*) and that this flavoprotein system may be replaced by the cytochrome system as growth proceeds.

It is interesting to note that respiration, relatively insensitive to cyanide, has also been demonstrated in resting spores of *Neurospora crassa* (Goddard & Smith, 1938) and in unfertilized *Arbacia* eggs (Korr, 1939). The latter were shown to contain haematin compounds but no cytochrome (Ball & Meyerhof,

1940), flavin-adenine dinucleotide (Krahl, Keltch & Clowes, 1940), and a substance resembling cytochrome oxidase (Krahl, Keltch, Neubeck & Clowes, 1941). A direct connexion between decreased cyanide-sensitivity and flavoprotein synthesis in yeast was reported by Pett (1935) who found that the flavoprotein content of this organism was doubled during growth in a medium containing cyanide. In this medium respiration was reduced, although fermentation remained at a normal level.

It seemed of interest, therefore, to determine the relative amounts of flavin-adenine dinucleotide in resting spores, germinated spores (Hills, 1950; Powell, 1950) and fully developed vegetative cells of *B. subtilis* and *Bacillus megatherium*, and to attempt to demonstrate the presence of enzymes containing flavin-adenine dinucleotide as their prosthetic group.

ORGANISMS AND METHODS

Organisms

Spores of the laboratory strain of *B. subtilis* used by Hills (1950) and of *B. subtilis* N.C.T.C. 85 were grown on CCY agar (Gladstone & Fildes, 1940) at 37°. They were reaped after a minimum of 3 days, washed five times with distilled water and stored at room temperature. Vegetative cells of the laboratory strain were grown on meat extract-peptone agar (Tarr, 1933) containing 1% Lab-Lemco, 1% peptone, and 0·5% NaCl, for 16 hr. at 28°. The cells were reaped and washed twice with large volumes of saline. No spores were visible in a stained film.

Spores of the laboratory strain require L-alanine specifically for optimal germination (Hills, 1950). During 30–60 min. incubation at 37° in a medium containing 5 mM-L-alanine and 50 mM-glucose buffered at pH 7·3 with 33 mM-phosphate, 90% of the spores in a 10^9/ml. suspension lose their heat resistance, becoming at the same time less refractile and more permeable to stains (Powell, 1950). Germinated forms for the present investigation were obtained in this way and were centrifuged and made up to a suitable concentration with distilled water.

A chemically defined medium in which spores of *B. subtilis* N.C.T.C. 85 will germinate has not yet been found, so that comparable results are only available for the resting spores of this organism.

Spores of a freshly isolated strain of *B. megatherium* were grown on meat extract-peptone agar (Tarr, 1933) containing 1% peptone, 0·2% Lab-Lemco, 0·5% NaCl and 0·2% glucose. They were reaped after 2 days at 37°, washed three times with distilled water, heated to 60° for 30 min. and finally washed twice more. It was found necessary to ice-cool suspensions of these spores until they had been washed several times, as inadequately washed suspensions germinated completely at room temperature (Powell, 1951). Vegetative cells were grown on CCY agar, reaped after 16 hr. at 28° and washed twice with saline. No spores were detectable in a stained film.

Germinated spores were obtained by incubating 10^9/ml. suspensions at 37° for 60 min. in 50 mM-glucose and 33 mM-phosphate buffer at pH 7·3. During this time about 90% of the spores showed the changes associated with germination (Powell, 1951) described above.

Methods

Determination of flavin-adenine dinucleotide by combination with the protein of D-*amino-acid oxidase.* The method described by Warburg & Christian (1938) was used. To facilitate extraction of flavin-adenine dinucleotide (FAD) the spores, germinated spores or vegetative cells were broken in a Mickle (1948) tissue disintegrator. A maximum of 12 ml. of a suspension containing 10–15 mg./ml. dry weight were shaken with 8 g. Ballotini beads, size 12, for 45–50 min., a drop of tributyl citrate being added to prevent foaming. After this treatment, very few intact cells could be seen in a stained film. Oxygen uptake was measured at 37° in Warburg vessels containing the broken cell material, an appropriate D-amino-acid, and an excess of D-amino-acid oxidase protein, in an atmosphere of oxygen. The system was buffered at pH 7·3 rather than at the optimum pH 8·8, since Krebs (1935) and Ochoa & Rossiter (1939) report a rapid fall in activity of the enzyme under alkaline conditions. Controls were set up in which the cell material was replaced by a crude preparation of FAD. Total FAD was determined on a portion of the broken cell suspension which had been heated to 100° and held at 75–80° for 10 min. in order to split the FAD from its enzyme proteins. Free FAD was determined on unheated samples.

Preparation of D-*amino-acid oxidase protein.* The apoenzyme of D-amino-acid oxidase was prepared from pig kidneys by a modification of the method of Negelein & Brömel (1939). Kidney cortex was minced and immediately freeze-dried. The freeze-dried material was then finely powdered and treated twice with large volumes of acetone. The acetone was removed by straining the tissue in a muslin bag, and then passing a stream of dry air through the powder for several hours. Although the freeze-dried material had quite high D-amino-acid oxidase activity before the acetone treatment, further purification omitting this step resulted in an entirely inactive preparation. The enzyme was extracted from the dried powder with 10 mM-phosphate buffer at pH 7·3 instead of the pyrophosphate used by Negelein & Brömel (1939), and the extract was treated as described in steps 1–5 of the above method. The final precipitate was freeze-dried and kept in a desiccator.

Preparation of flavin-adenine dinucleotide. The prosthetic group of D-amino-acid oxidase was prepared from baker's yeast (United Yeast Co.) according to the method of Warburg & Christian (1938) with a modification in the final stages suggested by the work of Straub (1938). The method was followed as far as the first precipitation of FAD with barium and the removal of the barium as barium sulphate. At this stage, the FAD was precipitated with a large volume of acetone (Straub, 1938), centrifuged, redissolved in a small volume of water and freeze-dried.

Absorption maxima for this preparation occurred at 260, 375 and 450 mμ. (Unicam spectrophotometer). Molecular extinctions at 375 and 450 mμ. were compared with those of the pure barium salt (Warburg & Christian, 1938) and indicated that the crude preparation contained not more than 10% (Table 1) of flavin-adenine dinucleotide, and probably rather less, since absorption by any free riboflavin present as an impurity was not taken into account.

An accurate estimate of the purity of this FAD preparation was obtained by comparison of its activity as the prosthetic group of D-amino-acid oxidase, with that of a sample of the barium salt of FAD (Warburg & Christian, 1938) containing only 13% of water. The conditions were the same as those used in the estimation of FAD in cell material. The weights of the two preparations were accurately determined on a microbalance and checked by measuring the extinctions of the solutions in the Unicam spectrophotometer at 450 mμ.

The molecular extinction coefficient ϵ is the optical density of a solution of concentration 1 g.mol./l. and path length 1 cm. Warburg & Christian (1938) measure the absorption coefficient, μ, which is the natural logarithm of the reciprocal transmission

$$\epsilon = \mu/(2{\cdot}3026 \times 10^3).$$

was transferred to a water bath maintained at 38°. The time needed for 90% reduction of the methylene blue was noted. Broken suspensions of resting and germinated spores and of vegetative cells, previously analysed for FAD were substituted for diaphorase and their activities compared. Some cell suspensions were also tested in the presence of added diaphorase, and of larger amounts of CoI (1 mg.). In a few experiments with broken spores the location of the enzyme activity was determined by testing the supernatant liquid and the cell debris separately after centrifuging.

Preparation of lactic dehydrogenase. The enzyme was prepared from ox heart by a modification of Straub's (1940) method. The heart muscle was minced, and the enzyme extracted with ice-cold water and precipitated by 60% saturation with ammonium sulphate. After centrifuging in the cold, the precipitate was dissolved in 10 mM-phosphate buffer, pH 7·3, and recentrifuged to remove insoluble material. A second precipitation at 60% saturation with ammonium sulphate gave a preparation which, when dissolved in a small volume of phosphate buffer, retained its activity during 2 months' storage at 4°. The enzyme solution was subsequently freeze-dried without loss of activity.

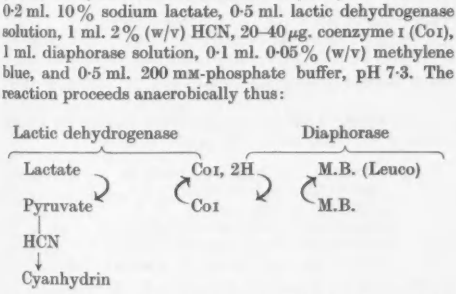
Table 1. *Spectrophotometric absorption data for flavin-adenine dinucleotide preparation*

Wavelength (mμ.)	260	375	450
Concn. of solution of crude FAD in μg. total solid/ml.	80	445	445
Extinction coefficient of crude FAD solution	1·3	0·50	0·535
Molecular extinction of crude FAD solution	$1{\cdot}34 \times 10^4$	$0{\cdot}103 \times 10^4$	$0{\cdot}111 \times 10^4$
Molecular extinction of pure barium salt (Warburg & Christian, 1938)	$3{\cdot}64 \times 10^4$	$0{\cdot}895 \times 10^4$	$1{\cdot}13 \times 10^4$
Percentage FAD in crude preparation	36·8	11·5	9·85

Detection of flavoproteins. L-Amino-acid oxidase, D-amino-acid oxidase and xanthine oxidase were assayed by measuring O_2 uptake, in an atmosphere of O_2, in the presence of the specific substrate.

For the detection of amino-acid oxidases, the system contained 16 mM-amino-acid, 40 mM-phosphate buffer at pH 7·3 and 10–20 mg. dry weight of cell material. Synthetic DL-alanine, valine, phenylalanine, norleucine and methionine were used. L-Leucine and L-proline were also tested at a concentration of 8 mM. To detect xanthine oxidase 3·3 mM-xanthine was substituted for the amino-acid.

The system used to detect diaphorase was that of Straub (1939; 1940) and Sumner & Krishnan (1948). It contained 0·2 ml. 10% sodium lactate, 0·5 ml. lactic dehydrogenase solution, 1 ml. 2% (w/v) HCN, 20–40 μg. coenzyme I (CoI), 1 ml. diaphorase solution, 0·1 ml. 0·05% (w/v) methylene blue, and 0·5 ml. 200 mM-phosphate buffer, pH 7·3. The reaction proceeds anaerobically thus:



diaphorase activity being indicated by decolorization of the methylene blue.

The method of Friedemann & Hollander (1942) was used in this test, because a qualitative result only was required. The results obtained cannot be regarded as quantitative owing to the presence of dissolved oxygen in the medium. An equal volume of 2% (w/v) melted agar, cooled to 45°, was added to the complete system contained in an ice-cooled test-tube. The contents of the tube were thoroughly mixed and when the agar had solidified (1 min.) the tube

Spore respiration. Oxygen uptake of spores was measured in Warburg manometers in 33 mM-phosphate buffer pH 7·3 at 37°, with and without the addition of 50 mM-glucose. In determinations of the cyanide-sensitivity of the respiration, steady overall concentrations of cyanide were maintained by the use of KOH-KCN mixtures in the centre well (Potter, 1948).

Chemicals. Coenzyme I (cozymase) was a commercial preparation supplied by L. Light and Co. Ltd., who also supplied DL-alanine, DL-valine, DL-phenylalanine, and DL-norleucine, and L-leucine and L-proline. L-Alanine was supplied by Roche Products Ltd. HCN 2% (w/v) was prepared freshly by neutralizing a KCN solution with H_2SO_4.

RESULTS

Flavin-adenine dinucleotide content of cells

A system containing 3 mg. D-amino-acid oxidase protein, 33 mM-DL-methionine, 2·7 μg. crude FAD and 75 mM-phosphate buffer (pH 7·3) in a total volume of 2·5 ml. gave an oxygen uptake of 53 μl./10 min. The rate of oxygen uptake was directly proportional to the amount of FAD added, provided

that the apoenzyme was in excess. With methionine as substrate the rate was four times as great as with DL-alanine. This activity ratio is twice that found by Krebs (1935) and is probably due to the presence of inhibitory substances in the synthetic DL-alanine, since another batch of this amino-acid inhibited the enzyme completely. Methionine was therefore generally used as substrate.

When disintegrated cells were substituted for crude FAD in the above system an oxygen uptake was recorded proportional to the amount of cell material added. Thus the relative amounts of free and combined FAD in spores, germinated spores and vegetative cells could be estimated. Compared with the pure barium salt of FAD, the preparation used as a standard contained $76{\cdot}8 \pm 3{\cdot}0$ mg. active FAD/g. The values given in Table 3 for the total FAD content of the cells examined are calculated from this estimation of the purity of the standard. The standard error quoted is that of the mean of two experiments from each of which quadruplicate results for both preparations were obtained.

The results of a typical experiment are summarized in Table 2. It was found that, in the absence of the substrate, or FAD, or the apoenzyme, the system was inactive. No oxygen uptake was recorded when cell material was substituted for FAD, unless both substrate and enzyme protein were present.

In a duplicated experiment, 40 μg. of FAD preparation were added to 10 ml. of spore suspension, the total and free dinucleotide activity then being measured in the usual way. The results indicated that none of the added FAD was destroyed during cell breakdown, but that when the total dinucleotide activity was determined after heating, there was a 30% fall in activity of the added material. On the other hand, when a broken spore suspension was heated for 30 min. at 75–80°, no fall in total dinucleotide activity was detected. It therefore seems probable that the partially purified FAD is more thermolabile than the FAD in the spore.

Experiments in which broken spore suspensions were centrifuged and the supernatant liquid substituted in the test system, showed that FAD activity was not associated with the cell debris. The addition of tri-butyl citrate, used to prevent foaming during cell breakdown, was found to have no effect on the oxygen uptake of the D-amino-acid oxidase system.

Table 2. *Protocol of experiment to assay flavin-adenine dinucleotide content of* Bacillus subtilis *spores*

	Controls (ml.)				Unheated spores (ml.)				Spores heated to 100° (ml.)			
Manometer	1	2	3	4	5	6	7	8	9	10	11	12
Water	1·25	1·25	1·5	1·0	0·5	0·75	0·25	0·75	0·5	0·75	0·25	0·75
Phosphate buffer, 200 mM, pH 7·3	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
DL-Methionine, 163 mM (in side arm)	Nil	0·25	0·25	0·25	Nil	0·25	0·25	0·25	Nil	0·25	0·25	0·25
Flavin-adenine dinucleotide, 8 μg. crude preparation/ml.	0·25	Nil	0·25	0·25	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
D-Amino-acid oxidase protein, 6 mg./ml.	0·5	0·5	Nil	0·5	0·5	Nil	0·5	0·5	0·5	Nil	0·5	0·5
Broken suspension of *B. subtilis* spores 5·5 mg./ml. (dry wt.)	Nil	Nil	Nil	Nil	1·0	1·0	1·0	0·5	1·0	1·0	1·0	0·5
20% (w/v) KOH (in centre well)	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Oxygen uptake (μl./10 min.)	0	0	0	40	0	0	21	10·5	0	0	41	20

Table 3. *Flavin-adenine dinucleotide content of bacterial cells*

(The content was estimated by measurement of the O_2 uptake of the unheated and heated suspension (=free and bound FAD) in presence of D-amino-acid oxidase apoenzyme + methionine. 1 ml. O_2/10 min. = 3·56 μg. FAD.)

	No. of experiments	Dry wt. of cells (mg.)	Spore count	Mean O_2 uptake of unheated suspension. Free FAD (μl./10 min.)	Mean O_2 uptake of heated suspension. Total FAD (μl./10 min.)	Percentage combined FAD	Total FAD (μg./g. dry wt.)
B. subtilis (lab. strain):							
Spores	5	5·56	10^{10}	22·2	42·2	48	29·4
Germinated spores	4	2·2 (approx.)	10^{10}	20·25	39·6	49	—
Vegetative cells	4	1·8	—	3·0	27·5	80	123·0
B. megatherium:							
Spores	11	7·76	10^{10}	9·3	59·0	84	29·7
Germinated spores	5	—	10^{10}	9·2	59·2	85	—
Vegetative cells	4	2·07	—	2·0	40·0	90	98·0
B. subtilis N.C.T.C. 85:							
Spores	3	7·35	$2{\cdot}2 \times 10^{10}$	52	54·5	5	28·9

The figures for FAD content of resting spores of two strains of *B. subtilis* and one strain of *B. megatherium* are given in Table 3. Although there were great differences in the ratio of free to combined FAD in the three organisms, the total amounts were remarkably similar.

Vegetative cells contained considerably greater amounts (3–4 times) of FAD. Comparisons were made on a basis of dry weight, which was determined by heating continuously at 104° for at least 48 hr. In germinated spores of the laboratory strain of *B. subtilis* and *B. megatherium*, both the total FAD and the ratio of free to combined FAD were the same as in resting spores on the basis of spore number. In this case, dry weight was not considered a satisfactory basis for comparison since germinated spores of *B. subtilis* were found to have a dry weight less than half that of the same number of resting spores, although no cell division had occurred. An explanation is at present being sought for this result. Slight inaccuracies must arise in this determination since after centrifuging the germinated spore suspension small amounts of medium still remain associated with the residue. Washing would have removed these, but might have introduced further errors, for example, by extracting soluble material from the germinated spore. The dry weight of germinated spores of *B. megatherium* was not determined.

Occurrence of flavoproteins

L-Amino-acid oxidase and D-amino-acid oxidase. No oxygen uptake generally occurred with resting spore suspensions of the laboratory strains of *B. megatherium* and *B. subtilis* in the presence of any of the amino-acids tested. In some cases in which a small oxygen uptake was recorded, it could be attributed to germination during the course of the experiment. Germinated spores possessed a low amino-acid oxidase activity (1 μl./hr./mg. dry weight) which was completely lost when the suspension was broken up.

Xanthine oxidase. Negligible rates of oxygen uptake were observed in the cells tested with xanthine as substrate. Vegetative cells of *B. subtilis* gave the highest oxygen uptake, which was only about 1 μl./hr./mg. dry weight.

Diaphorase. An enzyme similar in its action to the diaphorase of heart muscle was present in all cells of the three organisms studied. The enzyme remained in the supernatant liquid after the broken cell suspension had been centrifuged at $1100 \times g$ for 20 min. and was completely inactivated by heating for 5 min. at 100°. The diaphorase-like enzyme in resting and germinated spores of *B. megatherium* required the addition of much larger amounts of Co I for its demonstration than did that in the two strains of *B. subtilis*. It was later found that the activity of the heart diaphorase preparation was completely inhibited by broken spores of *B. megatherium*. The inhibition could be prevented by the addition of larger amounts (1 mg.) of Co I, but not by excess FAD. It seems that the spores of *B. megatherium* contain a substance which either removes, or competes with, Co I, under the experimental conditions. The inhibitor was associated with the cell debris and was stable during 10 min. at 100°. No such substance was present in the spores of *B. subtilis*, and moreover the action of the diaphorase-like enzyme which they contain was only slightly inhibited by addition of broken *B. megatherium* spores. This effect could not be accounted for by the presence of relatively large amounts of Co I in *B. subtilis* spores, since they were unable to reduce methylene blue when added to the dehydrogenase system in the absence of added Co I.

It may be significant that the times for reduction of methylene blue by the various cells were roughly in inverse proportion to the amount of bound FAD contained in these cells.

Oxygen uptake of spores

We have attempted to measure the oxygen uptake of resting spores of the laboratory strain of *B. subtilis* in phosphate buffer only. None could be detected using a micro-Warburg apparatus (Barker, 1949) containing up to 30 mg. dry weight of a ten-times washed suspension. A measurable, but variable, oxygen uptake occurred in the presence of 50 mM-glucose. This could be reduced by heating at 60° for 15 min., the Q_{O_2} over an experimental period of 2·5 hr. then being 0·42 compared with Q_{O_2} of 2·1 over the same period reported by Keilin & Hartree (1947) for spores of *B. subtilis* N.C.T.C. 85. The oxygen uptake of spores of the laboratory strain in 50 mM-glucose was found to increase 200-fold after 30 min. incubation in 5 mM-L-alanine. This high oxygen uptake, associated with germinated spores, was found to be considerably less sensitive to cyanide than that of vegetative cells. Thus 0·1 mM-HCN caused 54 % inhibition of vegetative cell respiration and 24 % inhibition of germinated spore respiration. Similar results were obtained with germinated spores and vegetative cells of *B. megatherium*. Here, 0·2 mM-HCN gave 70 % inhibition of vegetative cell respiration and 40 % inhibition of germinated spore respiration. Corresponding figures for 0·4 mM-HCN were 79 and 58 % respectively. These results imply that the oxygen uptake of resting spores may be even lower than that hitherto recorded, and that the cyanide-insensitive respiration previously considered to be a property of resting spores may be in fact due to a small proportion of germinated forms present in the suspensions.

DISCUSSION

Flavin-adenine dinucleotide and an enzyme closely resembling diaphorase in function have been found in all the resting spores, germinated spores and vegetative cells tested. Although the total amounts of flavin-adenine dinucleotide in spores of the three organisms investigated are very similar, the ratios of free to combined dinucleotide differ considerably. Diaphorase activity appears to run roughly parallel with the proportion of bound dinucleotide. These findings may not, however, represent the state in the intact spore, since it is possible that flavin-adenine dinucleotide is split from its apoenzyme to varying degrees in different organisms during cell breakdown.

During germination, i.e. the primary change involving loss of heat resistance of spores, no alteration could be detected in the total, or in the ratio of free to combined, flavin-adenine dinucleotide, when the comparison was made on the basis of spore number. Vegetative cells contained three to four times as much flavin-adenine dinucleotide per unit of dry weight and 80–90 % of this was in the combined state. They also had a much higher diaphorase activity than spores. A comparison between spores and vegetative cells based on dry weight must, however, be made with some caution, since the spore coat contributes an unknown proportion of the dry weight. Even with this reservation it does not seem that development from spore to vegetative cells is accompanied by a fall in flavin-adenine dinucleotide content or diaphorase activity. In view of the findings of Curran, Dewar, Gordon and Green (1939), indicating that xanthine oxidase can replace diaphorase in the dehydrogenase-methylene blue system, it is important to note that negligible xanthine oxidase activity was detected in the organisms tested.

It is significant that the respiration rate of spores of *B. subtilis* N.C.T.C. 85 was reported respectively by Cook (1931), Tarr (1933) and Keilin & Hartree (1947) as 90, 40 and 6 % of that of vegetative cells. It seems probable that the spore suspensions used contained germinated forms not easily characterized except by their lack of heat resistance and slight difference in staining properties (Powell, 1950). For instance, in the spore suspensions employed by Tarr (1933) fully developed vegetative cells were observed after 2 hr. incubation, so that, in this case at least, germination must have taken place at a considerably earlier stage in the experiments. Comparative insensitivity to cyanide cannot be taken as a criterion of resting spore respiration, since we have found that the oxygen uptake of germinated spores is also considerably less sensitive to cyanide than that of vegetative cells.

The occurrence of combined flavin-adenine dinucleotide, together with the demonstration of a diaphorase-like enzyme in spores of *B. subtilis* and *B. megatherium*, offers some support for the view that bacterial spores possess a respiratory mechanism involving a flavoprotein. We have found the oxygen uptake of resting spores in buffered glucose to be extremely low. If the uptake measured is, in fact, that of resting spores and not due to a small proportion of germinated forms, its cyanide-insensitive component may possibly be attributed to slow autoxidation of the diaphorase-like enzyme. If this enzyme is indeed diaphorase, it must be coupled with a readily autoxidized catalyst in germinated spores to account for their relatively high respiration rate. This catalyst may well be a haematin compound of comparatively low cyanide sensitivity.

In accounting for the low sensitivity of their respiration to cyanide, the possibility must not be ignored that spores may contain a large excess of heat-stable cytochrome oxidase (Militzer, Sondegger & Tuttle, 1950) over cytochrome *c* or some other intermediate constituent of the system. The amount of cytochrome *c* or other intermediate compound would then be the limiting factor in the oxidation, even when a large part of the cytochrome oxidase was inactivated by an inhibitor. Preliminary experiments to investigate this point have shown that both intact and broken resting spores of the three organisms used in this report will catalyse the oxidation of dimethyl *p*-phenylenediamine and quinol (Powell, 1952). These oxidations do not, however, show the characteristics of a typical cytochrome oxidase catalysis. Moreover, the oxidizing activity of spores disappears during 3–4 months' storage in water at room temperature, although viability and tendency to germinate in the chemically defined media are maintained. It seems possible, therefore, that an atypical cytochrome-cytochrome oxidase system may, at first, exist in resting spores, but that this system is not necessary to maintain viability or to promote the initial chemical changes involved in germination.

SUMMARY

1. Flavin-adenine dinucleotide has been shown to be present in all the cells tested, i.e. resting spores, germinated spores, and vegetative cells of laboratory strains of *Bacillus subtilis* and *B. megatherium*, and resting spores of *B. subtilis* N.C.T.C. 85.

2. During germination of spores of the laboratory strains of *B. subtilis* and *B. megatherium* in chemically defined media, no change in total flavin-adenine dinucleotide content, or in the relative amounts of free and bound dinucleotide occurred.

3. No L-amino-acid oxidase, D-amino-acid oxidase or xanthine oxidase activity could be demonstrated in resting spores of *B. subtilis* and *B. megatherium* or in germinated spores of *B. megatherium* and the laboratory strain of *B. subtilis*.

4. An enzyme capable of oxidizing reduced coenzyme I and reducing methylene blue occurred in all types of cell of *B. subtilis* and *B. megatherium*.

5. Spores of *B. megatherium* contained a heat-stable substance which inhibited reduction of methylene blue by heart diaphorase, the inhibition being prevented by the addition of large amounts (1 mg.) of coenzyme I.

6. No oxygen uptake could be measured with resting spores of the laboratory *B. subtilis* in phosphate buffer. The Q_{O_2} of these spores in buffered glucose, after preliminary heating at 60° for 15 min., was 0·42.

7. Oxygen uptake of *B. subtilis* and *B. megatherium* spores increased approximately 200 times following germination. This oxygen uptake was considerably less sensitive to cyanide than that of fully developed vegetative cells.

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Pseudo-Cholinesterase Activity in the Central Nervous System

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In an earlier report on the types of cholinesterases present in the tissues of the rat (Ord & Thompson, 1950), it was shown that whereas whole brain, striated muscle and suprarenal gland contain only small amounts of a 'non-specific' (Nachmansohn & Rothenberg, 1945) or 'pseudo'-cholinesterase (Mendel & Rudney, 1943*a*), the other tissues which were studied each showed a considerable level of activity of this type of esterase; stomach, liver, lung and salivary gland were found to hydrolyse benzoylcholine and acetyl-β-methylcholine at approximately equal rates, while heart, intestinal muscle and mucosa, Harderian gland and skin contain predominantly a pseudo-cholinesterase, benzoylcholine being hydrolysed from two to six times more rapidly than acetyl-β-methylcholine.

Although no evidence could at that time be brought forward as to the physiological significance of this widely distributed pseudo-cholinesterase, it was decided to extend this study to human tissues,

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and because of the interest attaching to the high level of overall cholinesterase activity in the central nervous system, a start was made with human brain.

Although little work has been done on human brain in this respect, the brains of a number of other mammalian species have received attention. Nachmansohn (1939) demonstrated the varying ability of different areas of dog, ox, rabbit and human brain to hydrolyse acetylcholine and showed that the white matter of the central nervous system was less active than the grey. Birkhaüser (1940) also reported on the acetylcholine-hydrolysing activity of human brain. Turning to the different types of cholinesterase, Mendel & Rudney (1943*b*), using 'specific' substrates for 'true' and pseudo-cholinesterases (Mendel, Mundell & Rudney, 1943), stated that the brains of a number of vertebrate species contained only the true cholinesterase and no pseudo-cholinesterase (see also Nachmansohn & Rothenberg, 1944). Little (1948), working with preparations derived from whole mouse brain, also failed to demonstrate any significant amount of pseudo-cholinesterase. Contrary to the earlier views on the specificity of brain cholinesterase, Augustinsson (1948) reported that extracts of dog and elephant brain and also of the caudate nucleus of the bear split benzoylcholine, although at a very low rate. Zeller (1949) also found both types of esterase present in elephant brain, and in homogenates of whole rat brain Ord & Thompson (1950) demonstrated a measurable rate of hydrolysis of benzoylcholine, although amounting to only about 6% of the rate of hydrolysis of acetylcholine. In a detailed study of the dog's brain Burgen & Chipman (1951) have recently shown that benzoylcholine is hydrolysed at a relatively rapid rate by certain areas. Both types of cholinesterase had earlier been found to be present in peripheral nerve (Boell, 1945; Sawyer, 1946).

In view of our earlier findings, and since the different types of cholinesterase do not appear to have been studied in human brain, we decided to investigate the substrate specificity of the cholinesterases in this human tissue, and to attempt to characterize the enzymes further by summation studies and by a study of the sensitivity of the hydrolysis of different esters to inhibition by eserine, di*iso*propyl fluorophosphonate and the selective inhibitor Nu 1250 (see below) introduced by Hawkins & Mendel (1949). We have also extended some of these findings to rat, rabbit, guinea pig, cat and dog brains.

EXPERIMENTAL

Estimation of esterase activity. This was carried out manometrically at 38° and at pH 7·4, using the Warburg apparatus. All measurements were made in duplicate, and were corrected for non-enzymic hydrolysis of the substrate. Esterase activity is expressed as μl. CO_2/g. wet wt. of tissue/hr.

Preparation of tissues. Human tissues were removed from cadavers as soon as possible after death; it was later found that storage of human nervous tissue at 0–4° for 3–5 days did not cause any significant loss of esterase activity.

The parts of the brain to be studied were dissected out, mopped free from adherent blood with filter paper, washed with 0·9% (w/v) saline, weighed and homogenized in 0·025 M-$NaHCO_3$. The homogenates, whose concentrations were chosen to give suitable CO_2 evolutions when measured over 0–30 min., were added to the main bulbs of the Warburg flasks.

Brains from other species were obtained after the animals had been killed by $CHCl_3$, decapitation or air-embolism; the areas under study were then prepared and homogenized as above.

Substrates. Acetylcholine chloride (ACh), British Drug Houses Ltd.; acetyl-β-methylcholine chloride ($[Me_3N^+CH_2.CHMe.OAc]\,Cl^-$) (MCh), Savory and Moore Ltd.; benzoylcholine chloride (BCh), prepared by Dr A. H. Ford Moore, Experimental Station, Porton; propionylcholine perchlorate (PrCh), provided by Dr V. P. Whittaker, Department of Biochemistry, Oxford, and later synthesized by standard methods; butyrylcholine chloride (BuCh), British Drug Houses Ltd.; tributyrin (TB), British Drug Houses Ltd.

The choline esters were dissolved in 0·025 M-$NaHCO_3$ immediately before use, the final concentrations used throughout being 0·015 M for ACh and BCh, and 0·03 M for MCh, PrCh and BuCh. The tributyrin was pipetted directly into the side arm of the Warburg flask (0·03 ml./flask), and was covered with a small volume of 0·025 M-$NaHCO_3$.

Inhibitors. Eserine sulphate, British Drug Houses Ltd.; di*iso*propyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton; *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250), Hoffman la Roche Ltd.

The inhibitors were dissolved in 0·025 M-$NaHCO_3$ immediately before use.

RESULTS

The relative activities of homogenates of different regions of human brain towards ACh, BCh, BuCh and MCh are shown in Table 1. The results given are the mean of at least two experiments with each tissue.

In agreement with the work of Nachmansohn (1939) and others, the rate of hydrolysis of acetylcholine varies widely according to the region studied, being high in the basal ganglia and other areas rich in synapses, but low in those parts consisting mostly of tracts of fibres.

When the activity towards acetyl-β-methylcholine is considered, it will be seen that those regions which are most active towards acetylcholine and which are rich in synapses (basal ganglia and grey matter) have also a high activity towards MCh, confirming the previous classification of brain cholinesterase as being mainly of the specific or 'true' type.

In all the regions studied, however, a measurable rate of hydrolysis of BCh was present. Here also variations in rate were found between preparations from different areas though the differences were smaller in degree than with either ACh or MCh. In regions rich in synapses, the activity towards BCh tended to follow the activity towards MCh, although it was in every case very much lower than the MCh level. On the other hand, in the different areas of subcortical white matter which were examined, the rate of hydrolysis of BCh was in every experiment greater than that of MCh, and amounted to 21–33 % of the activity towards ACh. This finding was emphasized when BuCh was used as a substrate instead of BCh. Although this compound is very readily hydrolysed by pseudo-cholinesterases it is only attacked slowly by the true cholinesterase. It will be seen from Table 1 that human subcortical white matter hydrolyses BuCh at more than twice the rate of ACh hydrolysis and more than seven times the rate for MCh.

Table 1. *Relative cholinesterase activities of different regions of human brain, using ACh, BCh, BuCh and MCh as substrates*

Region	Absolute activity (μl. CO_2/g. (wet wt.)/hr.)				Activity as percentage of ACh activity			Ratio:
	ACh	BCh	BuCh	MCh	BCh	BuCh	MCh	MCh/BCh
Lentiform nucleus	24650	438	1392	17775	2	6	72	36
Caudate nucleus	16900	240	881	10790	1	5	64	64
Substantia nigra	5540	173	—	4110	3	—	74	25
Cerebellum	4540	152	1600	2887	3	35	64	21
Thalamus	1620	94	650	942	6	40	58	10
Red nucleus	1537	128	—	1354	8	—	88	11
Cortical grey matter								
Pre- and post-central gyri	476	35	204	260	7	43	55	8
Frontal cortex	439	31	—	230	7	—	52	7
Occipital cortex	322	31	—	170	10	—	53	5
Subcortical white matter								
Pre- and post-central gyri	275	91	594	82	33	216	30	0·9
Frontal lobe	523	131	1024	64	25	196	12	0·5
Occipital lobe	319	66	—	53	21	—	17	0·8

Table 2. *Hydrolysis of tributyrin by different regions of human brain*

Region	Activity (μl. CO_2/g. (wet wt.)/hr.)
Lentiform nucleus	3972
Caudate nucleus	3393
Cerebellum	2132
Thalamus	2353
Cortical grey matter (pre- and post-central gyri)	2640
Subcortical white matter (pre- and post-central gyri)	802

A number of experiments have also been carried out with tributyrin as substrate in order to compare the rates of hydrolysis of non-choline esters by different regions of the brain with their cholinesterase contents (Table 2). The activity towards tributyrin was fairly constant in the areas of high synaptic density which were examined, but was much lower in the subcortical white matter. From a comparison with the results given in Table 1 the levels of TB hydrolysis do not appear to be related either to the activity towards MCh or towards BCh or BuCh. The independence of the enzymes hydrolysing these choline and non-choline esters is confirmed both by summation experiments (Table 3) and by the use of eserine as a differential inhibitor, 10^{-6} M-eserine sulphate producing 80–97 % inhibition of the hydrolysis of the choline esters, and only 2–22 % inhibition of the hydrolysis of TB (Table 4).

These results suggest that the white matter of the human cerebrum contains very much more pseudo-cholinesterase than the grey matter, although its tributyrinase content is lower. It was decided therefore to study the brains of other mammalian species to determine whether this predominant distribution of pseudo-cholinesterase in the white matter applies also to other species. Results obtained with preparations from the grey matter of the cerebral cortex and the subcortical white matter from five species are shown in Table 5; results obtained with human brain are included for comparison.

It will be seen that in all the species studied the rate of hydrolysis of BuCh by subcortical white matter was greater than that of MCh, and in every species except the dog BuCh was hydrolysed more rapidly by subcortical white matter than by the grey matter of the cerebral cortex.

Table 3. *Summation experiments with choline esters and tributyrin as substrates for human-brain preparations*

Enzyme source	Hydrolysis rate (μl. CO_2/g./hr.) Choline ester	Tributyrin	Both esters Found	Both esters Calc.	'Summation' (%)
	ACh				
Subcortical white matter	780	928	1496	1708	88
Cortical grey matter	585	3376	3580	3961	90
	MCh				
Cerebellum	3340	2660	5960	6000	99
	BuCh				
Subcortical white matter	817	1158	1813	1975	92
Lentiform nucleus	1202	4444	6180	5646	110
Caudate nucleus	938	3396	4620	4334	107

Table 4. *Effect of eserine* (10^{-6} M) *on the hydrolysis of choline esters and tributyrin by human-brain homogenates*

Enzyme source	Inhibition (%) Choline ester	Tributyrin
	BuCh	
Lentiform nucleus	82	8
Caudate nucleus	96	2
Subcortical white matter	80	8
	ACh	
Cerebellum	93	11
Cortical grey matter	97	22

Table 5. *Distribution of cholinesterases in the grey and white matter of the cerebrum of different mammalian species.*

Enzyme source	Absolute activity (μl. CO_2/g./hr.) ACh	BuCh	MCh	Activity as percentage of ACh activity BuCh	MCh	Ratio: MCh/BuCh
		Cortical grey matter				
Human	476	204	260	43	55	1·3
Rabbit	8340	1981	6710	24	81	3·4
Rat	6100	1935	4555	32	75	2·4
Guinea pig	4370	1009	2720	23	62	2·7
Cat	2340	117	2295	5	98	20
Dog	1799	1173	1063	65	59	0·9
		Subcortical white matter				
Human	275	594	82	216	30	0·1
Rabbit	7790	9084	2387	117	31	0·3
Rat	6525	3257	2389	50	37	0·7
Guinea pig	7145	18925	1564	266	22	0·1
Cat	2350	4550	549	194	23	0·1
Dog	328	595	151	181	46	0·3

Characterization of the pseudo-cholinesterase in white matter

Although BCh and BuCh have been shown to be 'specific' substrates for pseudo-cholinesterases from other sources it was thought advisable to characterize more exactly the enzyme in white matter hydrolysing these substrates.

Summation experiments with BCh and MCh confirmed that these substrates were hydrolysed by different enzymes; for example, the rates of hydrolysis found in one experiment with human white matter were 64, 97 and 139 μl. CO_2/g./hr. for BCh, MCh and BCh+MCh respectively, i.e. 87% 'summation' was obtained.

As the white matter in all the species studied

always showed considerable true cholinesterase activity, a preparation from human subcortical white matter was partially purified by centrifugation of a water homogenate, the greater part of the true cholinesterase being discarded in the centrifuged supernatant (Ord & Thompson, 1951). Table 6 gives a comparison of the relative rates of hydrolysis of a number of different esters by the unpurified and partially purified preparations from white matter with those found by Adams & Whittaker (1949) for the purified cholinesterase of human plasma.

Table 6. *Comparison of the relative rates of hydrolysis of different esters by human white matter (cerebrum) and by purified human plasma pseudo-cholinesterase*

Substrate	Human white matter: Unpurified	Human white matter: Partially purified	Human plasma*
Acetylcholine	100	100	100
Benzoylcholine	33	31	36
Propionylcholine	157	160	—
Butyrylcholine	216	202	208
Acetyl-β-methylcholine	30	12	1
Tributyrin	293	—	45

* Data of Adams & Whittaker (1949).

The relative rates of hydrolysis of ACh, BCh and BuCh agree well with those found for the purified plasma enzyme, although the unpurified brain preparation naturally hydrolyses MCh and TB very much more rapidly. The similarity between the BCh-hydrolysing enzyme in white matter and the plasma pseudo-cholinesterase is further borne out when the concentrations of DFP required to inhibit the enzyme in these two sources are compared (Table 7). This table also shows that the sensitivity of the MCh-hydrolysing enzyme to inhibition by DFP closely resembles that of the true cholinesterase in human erythrocytes, and provides further evidence for the presence of these two types of cholinesterase in white matter.

Table 7. *Concentrations (I_{50}) of DFP required to produce 50% inhibition of the cholinesterases of human white matter, plasma and erythrocytes*

Enzyme source	Substrate	I_{50} ($\times 10^{-9}$ M)
Human white matter	BCh	2·0
Human white matter	BuCh	1·4
Human plasma*	BCh	2·6
Human white matter	MCh	125
Human erythrocytes*	MCh	130

* Data of Adams & Thompson (1948).

The finding that, of the five choline esters studied (Table 6), butyrylcholine is hydrolysed most rapidly suggests that this enzyme in human subcortical white matter may be of the butyro-cholinesterase type (Sturge & Whittaker, 1950), very similar in those of its properties which have been examined to the pseudo-cholinesterase in plasma. In the white matter of rat brain the MCh/BuCh ratio was significantly higher than that obtained for the other species studied (Table 5). Since we had earlier (Ord & Thompson, 1951) found that the purified pseudo-cholinesterase in rat heart is a 'propiono-cholinesterase', hydrolysing PrCh almost twice as rapidly as BuCh, we tested a rat-brain preparation (white matter) with PrCh; we found that the PrCh/BuCh ratio was 3·2, indicating that the predominant cholinesterase in this rat tissue may also be of the propiono-cholinesterase type rather than the butyro-cholinesterase type present in the other species.

Quantitative aspects of the distribution of cholinesterases in human brain

Since the different regions of the brain which we have studied all show activity towards both BCh and MCh, any quantitative consideration of the relative amounts of pseudo-cholinesterase in the different areas is complicated by the fact that the level of the true cholinesterase varies so very widely in different regions. It is for this reason that detailed consideration has so far been restricted to the grey and white matter of the cerebrum, in each of which areas the overall activity towards ACh is of the same order. It will have been noticed, however (Table 1), that the levels of hydrolysis of BCh and BuCh are very high indeed in the basal ganglia, cerebellum and thalamus. Moreover, in these regions the activity towards these substrates tends to parallel that towards MCh. Preparations from the caudate nucleus of the rabbit and the rat were also studied, and in these species also a high level of true cholinesterase was found to be associated with a high rate of BuCh hydrolysis.

These facts suggested that the very large amount of true cholinesterase in these areas might be contributing to the hydrolysis of BCh and BuCh. Adams (1949) has reported that the purified true cholinesterase from human erythrocytes hydrolyses BuCh and BCh at 5 and 1·5% respectively of the rate at which it hydrolyses MCh. If this also applies to the true cholinesterase in brain it would indicate that a considerable contribution to the high rate of hydrolysis of BuCh by these areas would be due to the splitting of this substrate by the very high concentration of true cholinesterase present.

Experiments were therefore carried out with the selective inhibitors DFP and Nu 1250 to compare the sensitivity of BuCh hydrolysis to these compounds in a region poor in true cholinesterase (subcortical white matter) with regions very rich in this enzyme

(lentiform and caudate nuclei and cerebellum). The results of these experiments are given in Table 8.

It will be seen that the hydrolysis of BuCh by subcortical white matter is inhibited 96 and 8% respectively by these concentrations of DFP and Nu 1250, indicating that in this region the hydrolysis is almost entirely brought about by an esterase sensitive to DFP but insensitive to Nu 1250, i.e. a pseudo-cholinesterase. In the areas very rich in true cholinesterase, however, BuCh hydrolysis is only partially inhibited (36–51%) by either of these

Table 8. *Inhibition of human-brain cholinesterases by DFP and Nu* 1250

Tissue	Substrate	Inhibition (%) produced by $0{\cdot}9 \times 10^{-8}$ M DFP	Inhibition (%) produced by $1{\cdot}67 \times 10^{-6}$ M Nu 1250
Subcortical white matter	MCh	3	100
	BuCh	96	8
Lentiform nucleus	MCh	18	97
	BuCh	36	50
Caudate nucleus	MCh	10	98
	BuCh	51	45
Cerebellum	MCh	9	—
	BuCh	49	—

two compounds. Since the concentrations of the two inhibitors which we have used are sufficient to cause almost complete inhibition of the pseudo- and true cholinesterases respectively, these results suggest that in these regions BuCh hydrolysis is being brought about to a significant degree by both enzymes, so that a measure of its rate of hydrolysis by these parts does not give a valid assessment of the amount of pseudo-cholinesterase present.

DISCUSSION

The results described above indicate that all the areas of human brain which have been studied contain, in addition to their true cholinesterase, an enzyme that hydrolyses both BCh and BuCh but not MCh. From its substrate specificity and from studies of sensitivity to inhibition by eserine and DFP it is clear that this enzyme is distinct from the tributyrinase which is present in these preparations, and is similar to, if not identical with, the pseudo-cholinesterase present in human plasma.

The distribution of this enzyme in the different areas of the brain raises some interesting points. The absolute level of BuCh hydrolysis is high in the lentiform and caudate nucleus, the cerebellum and the thalamus; but, as pointed out above, it would seem that a considerable part of this activity is due to hydrolysis of BuCh by the relatively enormous concentration of true cholinesterase present in these parts. However, a comparison of the grey and white matter of the human cerebrum, areas having ACh-hydrolysing activities of the same order, shows that the subcortical white matter has very much greater pseudo-cholinesterase activity than the grey; the extremely low level of true cholinesterase activity in this region indicates that it is not playing any significant part in the BuCh hydrolysis. In each of the other species studied, the level of pseudo-cholinesterase is also considerably greater than that of the true cholinesterase in the white matter, and in every species except the dog it is also present in much larger amounts in the subcortical white matter than in the cortical grey. The high level of this enzyme in the white matter of the cerebrum suggests that it may be associated particularly with the myelinated fibres of the nervous system, and experiments are now in progress with spinal cord and with myelinated peripheral nerves to determine whether this association holds also for the myelin-containing regions of these tissues. It is of interest that Burgen & Chipman (1951), in their recent study of dog brain, have concluded that in this species also the fibre tracts in general have a high BCh-hydrolysing activity.

These findings naturally raise the question of the function of this pseudo-cholinesterase, particularly in the white, myelinated areas of the central nervous system. One possibility is that it may be connected with processes occurring in the myelin sheaths or the neuroglial elements of the nervous system rather than directly with the conducting properties of the axis cylinders. Support is given to this suggestion by the findings of Sawyer (1946), who reported that Wallerian degeneration of guinea pig peripheral nerves, with complete disintegration of the axis cylinders, is not associated with any loss of pseudo-cholinesterase activity in the affected nerves, even though the true cholinesterase falls by about 60%. In this connexion it may be of interest to record some preliminary observations which we have made with tri-*o*-cresyl phosphate (Earl & Thompson, 1952). This substance was shown by Smith & Lillie (1931) to produce areas of demyelination both in the spinal cord and in peripheral nerves. Bloch in 1941 had demonstrated *in vitro* its anti-cholinesterase properties. Despite this latter finding, injection of the compound into animals does not, in most of the species studied, give rise to any signs of acetylcholine accumulation. Tri-*o*-cresyl phosphate is, however, only very slightly soluble in water, although it is freely lipoid-soluble, and together with Dr C. J. Earl we have shown that it is a highly selective inhibitor for the pseudo-cholinesterase in human brain and plasma; *in vitro* we have found that concentrations of $1{\cdot}4 \times 10^{-4}$ M cause 60–90% inhibition of the pseudo-cholinesterase in human cerebrum, but only 0–7% inhibition of the true cholinesterase in this site or in human erythrocytes. It

must be stressed that these latter findings are of a preliminary nature and are as yet unsupported by any *in vivo* work, so that although we have evidence of an association of pseudo-cholinesterase activity with the white fibre tracts of the central nervous system we are at present unable to reach any conclusions as to its physiological function in these areas.

SUMMARY

1. The relative rates of hydrolysis of a number of different choline esters by preparations from different areas of human and other mammalian brains have been determined.

2. In all the areas studied, a measurable degree of hydrolysis of benzoylcholine and butyrylcholine was observed.

3. The benzoylcholine-hydrolysing enzyme present in human nervous tissue appears to be a 'butyro-cholinesterase' closely resembling the pseudo-cholinesterase present in human plasma.

4. The white fibre tracts of the human cerebrum were found to contain significantly more of this pseudo-cholinesterase than the grey matter.

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The Metabolism of Short-Chain Fatty Acids in the Sheep

1. FATTY ACID UTILIZATION AND KETONE BODY PRODUCTION BY RUMEN EPITHELIUM AND OTHER TISSUES

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Large quantities of volatile fatty acids, chiefly acetic, propionic and butyric acids, are produced by the bacterial decomposition of the foodstuffs in the rumen of the sheep and other ruminants and are absorbed directly from this organ (see review by Elsden & Phillipson, 1948). The problem of assessing the relative amount of each acid produced is not altogether straightforward, owing to differing rates of absorption. Application of chromatography has led to the identification of smaller quantities of higher isomers and of formic acid (Gray, Pilgrim, Rodda & Weller, 1951). Subsidiary quantities of volatile fatty acids are also produced in and absorbed from the large intestine.

Although the production of volatile fatty acids in the gut is not confined to ruminants (Phillipson, 1947*a*), the latter present a special problem since it is highly probable that these compounds, previously considered as by-products of digestion, form their chief source of energy. The amount of sugar available

for absorption appears to be very small; it is unlikely that much escapes fermentation in the gut. McClymont & Scarisbrick (unpublished data, quoted by McClymont, 1951) obtained evidence suggesting that at least on a diet of lucerne hay and green kale the amount of reducing sugar available to a sheep from intestinal enzymic digestion is equivalent to only 1–3 g. of glucose per day. The level of volatile fatty acid in the peripheral blood of the sheep is up to about 10 mg./100 ml. (Schambye & Phillipson, 1949; Reid, 1950), which is many times greater than that reported for the dog and man (McClendon, 1944). Conversely, the blood glucose level in adult sheep does not normally exceed 50 mg./100 ml. (Schambye & Phillipson, 1949; Reid, 1950); this is only about one-half that found in most mammals.

Obviously the study of the subsequent fate of the volatile fatty acids is a major problem in ruminant physiology. Few investigations have yet been reported in this field, although numerous studies with the usual laboratory animals have shown that some animal tissues are capable of utilizing these substances, which cannot be stored in the body to an appreciable extent, unlike carbohydrates or the higher fatty acids. Acetic acid is now looked upon as an important metabolic intermediate (Bloch & Rittenberg, 1945) and a precursor of a wide variety of compounds in the body, including higher fatty acids and cholesterol. It can be converted to ketone bodies in the liver. Butyric acid is known to be strongly ketogenic in liver tissue, by which it is readily metabolized. Propionic acid, on the other hand, can give rise to extra carbohydrate; in ruminants, whose supply of carbohydrate is otherwise restricted, this latter fact is probably of considerable importance. Jarrett & Potter (1950) administered acetate and propionate to sheep and found that acetate increased the level of ketone bodies in the blood whilst propionate decreased the ketone bodies and raised the levels of pyruvic and lactic acids. There was little rise in ketone bodies when acetate and propionate were administered together, and they suggest that the oxidation of acetate is linked with that of propionate. Schultz, Smith & Lardy (1949) could detect no increase in blood ketone bodies after administration of acetic acid to goats, although butyric and some higher acids were ketogenic.

The starting point of the work described in the present paper was the observation (Masson & Phillipson, 1951; Kiddle, Marshall & Phillipson, 1951) that the amount of butyric acid in the blood draining the sheep's rumen was less, relative to the level of acetic acid, than the relative amount disappearing from the rumen, a fact which pointed to a utilization of butyrate in the rumen wall. This was probably not simply an esterification, since McClymont (1951) could find no significant level of esterified lower fatty acids in bovine blood lipids. The *in vitro* metabolism of acetic, propionic and butyric acids by the rumen epithelium has now been investigated in detail and studies have been made with other sheep tissues.

EXPERIMENTAL

Tissues were obtained from sheep killed at the slaughterhouse. They were removed immediately into partly frozen Krebs-Ringer solution (Umbreit, Burris & Stauffer, 1945), and the experiments were normally commenced after approximately 2 hr. Tissue sections were prepared as described below, collected in ice-cold Krebs-Ringer, pooled, gently blotted with filter paper and a weighed amount (usually 2 g.) incubated in 50 ml. conical flasks with 10 ml. of Krebs-Ringer-phosphate, pH 7·2 (Umbreit *et al.* 1945) containing 100 μmoles of the sodium salt of one of the fatty acids. The flasks, after filling with O_2, were shaken (105 strokes/min.) in a water bath at 39·5° for 3 hr. Any variation in this procedure is stated in the text and tables. Following incubation the tissue was removed and washed twice with distilled water. The dry weight of the tissue was determined by drying at 105° to constant weight. The medium and washings were combined and made up to 25 ml. For determination of the residual volatile fatty acid 1 ml. of 10 N-H_2SO_4 and 8 g. of $MgSO_4.7H_2O$ were added to 10 ml. of this liquid, followed by steam distillation in a Markham still. A sample of the distillate (100 ml.) was titrated with 0·01 N-NaOH under CO_2-free conditions; each determination was carried out in duplicate. The addition of $MgSO_4$ served not only to increase the rate of distillation of the fatty acid, but also to hold back HCl, which otherwise came over in appreciable quantities. It was found by preliminary tests that approximately 5% of the unused fatty acid was retained by the tissue, even after washing, and all the results obtained were corrected by this amount.

Volatile fatty acid in blood was determined by the procedure of McClendon (1944) with the slight modification due to Phillipson (1947*a*).

Ketone bodies were determined on the tissue medium and in blood by the method of Greenberg & Lester (1944). The dinitrophenylhydrazine reagent was dissolved in 0·5 N-HCl instead of 2 N suggested by the authors; this obviated the high blanks which were otherwise sometimes encountered. Also, it was found that rapid shaking for 10 min. was not always adequate for extraction of the colour into the CCl_4 and 15 min. were allowed. A further quantity of ketone bodies, amounting to approximately 10% of the quantity in the medium, could be extracted from the tissue sections by boiling with water under reflux after separation from the medium and washing. All values were corrected for this factor.

In view of the recognized difficulties in the accurate determination of ketone bodies it was felt advisable to compare the method with a different one. The procedure of Weichselbaum & Somogyi (1941) was applied to two filtrates following incubation of rumen epithelial tissue in the butyrate medium and to a sample of arterial blood from a normal sheep. The results were respectively 38·5 and 48·3 μmoles and 21·8 μmoles/100 ml.; the results obtained on the same samples with the Greenberg & Lester method were 47·5 and 44·2 μmoles and 23·3 μmoles/100 ml.

RESULTS

In some preliminary experiments the disappearance of volatile fatty acids when incubated with slices cut from the rumen wall was measured. Table 1 shows the results of experiments with material from three sheep. It is evident that all three acids were metabolized under these conditions and that there was a marked preference for butyrate; this confirms the conclusion drawn from the absorption studies mentioned above. The stability of the systems responsible, i.e. no measurable loss of activity after several hours in the cold (sheep 1, Table 1), increases confidence that the activity of the rumen wall towards these substances would not be markedly different *in vivo*.

Table 1. *Uptake of volatile fatty acids by sheep rumen slices*

(Ringer-phosphate, pH 7·2, containing 100 μmoles of fatty acid as Na salt. 1 g. (wet wt.) of tissue in sheep no. 1, 2 g. in sheep nos. 2 and 3.)

Sheep no.	Duration (hr.)	Temperature (°)	Fatty acid lost (μmoles) Acetic	Propionic	Butyric
1	3	37·5	5·3	4·6	18·1
			6·1*	4·1*	19·0*
2	4	34·0	19·0	15·3	38·3
3	3·5	37·5	18·2	11·1	62·9

* Slices first kept for 6 hr. in ice-cold Ringer.

Table 2. *Uptake of volatile fatty acids by rumen epithelium and rumen muscle*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt.)

Sheep no.	Fatty acid lost (μmoles) Epithelium Acetic	Epithelium Propionic	Epithelium Butyric	Muscle Acetic	Muscle Propionic	Muscle Butyric
1	17·2	10·0	92·5	9·8	8·3	19·2
2	22·7	28·5	79·0	8·3	6·8	11·0

Since the rumen wall comprises predominantly two separate types of tissue, the stratified squamous epithelium and muscle, the activity of each was next studied separately (Table 2). This was easily accomplished by peeling off the epithelium and slicing the muscle in the usual manner. It was unnecessary, and indeed difficult, to slice the epithelium; it was simply cut into pieces about 1 cm. square. Such preparations contained connective tissue in addition to the epithelial cells, but were free from muscle, as shown by histological examination. Rumen epithelium is an ideal tissue for *in vitro* metabolic studies, being thin and tough, with a large surface area owing to its papillae; moreover, large quantities can be prepared in a very short time.

It can be seen from the figures that the epithelium was considerably more active than the muscle, although it is not known to what extent the greater tissue damage necessarily involved in the preparation of the muscle slices is responsible for this. A further point is that the relative rate of disappearance of butyrate was greater with the epithelium than with the muscle tissue. In subsequent experiments epithelium alone was used. Table 3 shows the results obtained with material from ten sheep; it gives an idea of the extent of the variation in the ability to utilize each fatty acid. In a further experiment, four parallel runs with butyrate using epithelium from the same animal gave results of 26·2, 27·2, 27·1 and 27·6 μmoles.

Table 3. *Uptake of volatile fatty acids by samples of rumen epithelium from ten sheep*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt. The figures in this and subsequent tables (4–8) are calculated to 100 mg. (dry wt.) of tissue.)

Fatty acid lost (μmoles) Acetic	Propionic	Butyric	Volatile acid production in control (μmoles)
5·2	7·0	25·1	2·6
5·6	7·8	25·3	0·7
4·0	2·2	29·5	2·9
5·6	10·4	28·9	1·8
4·9	8·6	30·1	1·1
8·0	19·0	36·0	0·3
2·1	1·5	15·3	1·2
4·5	10·6	26·3	1·5
4·7	4·6	29·4	1·7
4·1	3·2	29·3	2·7
Mean 4·9	7·5	27·5	1·6

The figures in the fourth column of Table 3 represent the volatile acid production in control flasks containing no added fatty acid; the figures in

the other columns have been corrected by the addition of the figures in column 4. In this and in subsequent tables the amounts of fatty acid utilized are calculated to 100 mg. of tissue (dry weight) in order to eliminate the effect of any variations in the wet weight/dry weight ratio. Such variations never exceeded 10 % of the ratio in any one experiment. The loss of dry weight during incubation of the tissue amounted to less than 10 %, so that the figures are slightly higher than if based on initial dry weight.

It is clear that in every experiment the disappearance of butyrate exceeded that of the other two.

Table 4. *Influence of* pH *of medium upon uptake of volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt.)

	Fatty acid lost (μmoles)			Volatile acid production in control
pH	Acetic	Propionic	Butyric	(μmoles)
7·2	4·9	8·6	30·1	1·1
6·5	5·6	2·1	16·1	1·0
5·8	3·9	0·5	10·3	0·5

The activity of the tissue appeared to be well maintained during the 3 hr. incubation, since when incubated for only 1 or 2 hr. the amount of acid disappearing was correspondingly less. A longer incubation period was not used owing to the risk of bacterial growth in the flasks.

Some experiments were carried out in which the conditions of incubation were altered. When the concentration of fatty acids was increased eightfold the amounts disappearing were increased 3–5 times. Table 4 shows the effect on the disappearance rates of lowering the pH of the medium by the addition of hydrochloric acid. (The additional chloride thus introduced was very small compared to that already present in the medium and would not, in itself, be expected to influence the results.)

The effect of using bicarbonate buffer of approximately the same pH (Krebs-Ringer-bicarbonate, Umbreit *et al.* 1945) instead of phosphate buffer, in an otherwise identical medium, was also determined. Ciaranfi (1936) found a more rapid utilization of butyric acid by liver slices in a phosphate-buffered than in a bicarbonate-buffered medium. The results of three experiments with different sheep are shown in Table 5.

Table 5. *Influence of buffer and of carbon dioxide upon uptake of volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate or Ringer-bicarbonate containing 100 μmoles of fatty acid as Na salt.)

Sheep no.	Buffer	Gas phase	Fatty acid lost (μmoles) Acetic	Propionic	Butyric
1	Phosphate	O_2	5·6	7·8	25·3
	Bicarbonate	O_2+5% CO_2	5·0	13·7	24·6
2	Phosphate	O_2	5·6	10·4	28·9
	Bicarbonate	O_2+5% CO_2	3·6	20·3	29·9
	Phosphate	O_2+5% CO_2	6·5	18·8	31·4
3	Phosphate	O_2	4·4	1·2	27·3
	Phosphate	O_2+5% CO_2	6·0	6·0	27·1

Although the mean of the propionate figures is greater than that of the acetate, this is of little importance owing to the very great variation among the propionate results. (It is of interest that the variance of the latter is 26·98, compared to 2·24 for acetate; a variance ratio of 12 indicates a highly significant difference ($P = 0{\cdot}002$) between the two variances.)

In one of the experiments the rates of distillation of the unused acids were recorded by collecting the distillates in 10 ml. fractions and titrating each fraction. In each case the observed rate corresponded exactly with that of an equivalent quantity of the original fatty acid, thus providing a check that the estimated volatile acid in the experiments represented entirely the unused portion of the original 100 μmoles and not a different volatile acid produced from it by the tissue.

In the first experiment the substitution of bicarbonate for phosphate had a striking effect upon the utilization of propionate, the disappearance rate of which was nearly doubled. This effect was confirmed in the second experiment, where, in addition, the disappearance rates were determined using phosphate buffer but gassing the flasks with oxygen + 5 % of carbon dioxide, as used with the bicarbonate buffer. The elevation in the rate of disappearance of propionate was maintained. In the case of the other two fatty acids there were no such marked differences resulting from the presence of carbon dioxide. The relatively small effect upon acetate utilization resulting from the presence of carbon dioxide may not be significant. This will be investigated further.

The important question of the fate of the fatty acids metabolized by the rumen epithelium was

partly answered by testing the media, after incubation, for ketone bodies. Application of Rothera's test invariably gave a strong response following the disappearance of butyrate. A positive response was obtained also on the acetate media but none in the case of propionate, although the controls incubated without fatty acid always gave a little colour. In two subsequent experiments determinations of ketone bodies were carried out (Table 6: results with two sheep).

Table 6. *Production of ketone bodies from volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.)

	Ketone bodies produced (μmoles) in presence of		
Acetic	Propionic	Butyric	Control
1·8 (76)	−2·7	22·7 (77)	4·4
0·4 (19)	−2·7	18·9 (65)	3·3

The figures (calculated as usual to 100 mg. dry weight) are corrected for the ketone production in the controls. The amount of fatty acid utilized was also determined (nos. 9 and 10 in Table 3) and the proportion of this appearing as ketone bodies calculated (assuming that one molecule of ketone is derived from two molecules of acetic acid or one molecule of butyric acid). It can be seen that more than one-half of the butyric acid disappearing could be accounted for as ketone bodies. Of the total ketone body production less than one-quarter was present as β-hydroxybutyric acid. In the case of acetate the relatively small difference from the controls makes the validity of the calculated percentages doubtful, since it is impossible to say whether the endogenous reactions producing ketone bodies in the controls would continue at the same rate in the presence of added substrate. If not, the corrections are not justified and the figures should consequently be higher. The appearance of ketone bodies in the propionate medium was in each case less than that in the control medium.

In another experiment the initial concentration of butyrate was halved (50 μmoles/flask). The proportion of that lost appearing as ketone bodies was 65 % as compared to 70 % in a parallel run employing the usual concentration of butyrate. In the experiment employing eightfold concentrations of the fatty acids, mentioned above, 59 % of the metabolized butyric acid was converted to ketone bodies.

In further experiments the ability of other tissues of the sheep to utilize the same fatty acids under identical conditions was determined; the ketone body production was measured in each case. The results are summarized in Table 7. Both fatty acid disappearance and ketone production are corrected by the control figures, as in previous tables.

Table 7. *Volatile fatty acid uptake and ketone body production by various sheep tissues*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt. Results given as μmoles. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.)

	Acetic		Propionic		Butyric		Control	
Tissue	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Volatile acid produced	Ketone bodies produced
Reticulum epithelium	5·3	1·5 (56)	3·9	−0·3	23·8	17·5 (73)	1·3	0·6
	2·5	0·2 (16)	1·5	−0·2	10·8	5·3 (49)	0·3	0·5
Omasum epithelium	4·6	1·9 (83)	1·4	−0·8	30·0	24·2 (81)	0·0	1·7
	1·6	0·3 (38)	0·7	−0·2	8·6	4·0 (47)	0·8	1·0
Abomasum epithelium	18·8	−0·4	14·2	−0·4	18·2	3·3 (18)	0·2	0·9
	20·2	2·2 (22)	13·7	0·5 (8)	16·9	5·3 (31)	0·0	0·4
Caecum epithelium	12·6	0·4 (6)	10·0	0·2 (4)	9·4	1·1 (12)	1·0	0·3
	10·8	−0·9	6·7	−0·9	10·0	1·0 (10)	1·2	1·8
	13·0	0·3 (5)	8·8	0·0	11·6	0·3 (3)	0·7	1·2
Liver	6·9	0·9 (26)	5·9	0·6 (20)	20·0	14·8 (74)	0·6	0·0
	15·8	1·6 (20)	22·3	−3·8	23·9	16·6 (69)	0·9	1·9
Kidney cortex	42·6	0·0	43·8	0·0	38·5	3·8 (10)	0·0	1·3
	54·5*	0·6 (2)	60·9	0·0	41·2	8·6 (21)	0·0	0·4
Heart muscle	0·0	—	0·0	—	1·4	0·1 (7)	0·4	0·2
	4·2	0·1 (2)	0·4	0·0	1·1	0·2 (18)	0·6	0·0
Brain	0·0	—	0·0	—	0·0	—	1·2	—
	0·0	—	0·0	—	0·0	—	2·1	—

* Only 1 g. of tissue used.

The figures in each line in the table were obtained on tissue from a different sheep. The epithelial tissues were prepared in the same way as the rumen epithelium; although this was rather difficult in the case of epithelium from the abomasum and caecum owing to their greater fragility, satisfactory results were obtained after a little practice. The other tissues were sliced in the standard manner. As a ketone bodies. On the other hand, kidney cortex, and epithelium from the abomasum (the true stomach of the sheep) and from the caecum, utilized acetate at least as rapidly as butyrate and, moreover, produced a relatively smaller amount of ketone bodies. The results with heart-muscle slices were low and less consistent. Slices of whole brain did not utilize any of the three fatty acids.

Table 8. *Volatile fatty acid uptake and ketone body production by rat tissues*

(Liver tissue (2 g.) or 1 g. of kidney tissue incubated for 3 hr. at 39·5° in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt. Results given as μmoles. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.

	Acetic		Propionic		Butyric		Control	
	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Volatile acid produced	Ketone bodies produced
Liver	27·0	3·1 (23)	23·1	−2·1	31·5	19·6 (62)	0·7	7·7
Kidney	92·5	1·2 (3)	56·8	0·9 (3)	58·0	6·0 (10)	0·9	0·0

Table 9. *Changes in blood volatile fatty acids and ketone bodies resulting from the administration of fatty acids into the rumen*

(Results expressed as μmoles/100 ml.)

	Rumen blood		Carotid blood	
Liquid in rumen	Ketone bodies	Fatty acid	Ketone bodies	Fatty acid
Exp. 1				
Water	48	77	58	27
Sodium butyrate, 0·15 M	222	118	199	73
Water	83	46	86	41
Sodium propionate, 0·15 M	83	369	86	100
Water	60	46	58	50
Sodium acetate, 0·15 M	78	611	76	114
Water	19	68	48	45
Sodium butyrate, 0·15 M	219	123	191	64
Exp. 2				
Water	47	50	47	50
Sodium butyrate, 0·15 M	310	375	266	146
Water	110	32	95	27
Sodium propionate, 0·15 M	43	565	43	100
Water	24	27	43	27
Sodium acetate, 0·15 M	100	1088	47	142
Water	33	32	47	36
Sodium butyrate, 0·15 M	195*	320*	134*	96*
	334	246	248	50

* Blood samples taken after 5 min. All other samples taken 30 min. after administration.

check, in each case, rumen epithelium from the same sheep was incubated with butyrate; in every case the amount of butyrate utilized fell within the range of values for butyrate seen in Table 3 and the proportion converted to ketone bodies ranged from 59 to 74%. Inspection of Table 7 shows that epithelium from the reticulum and omasum and also liver tissue behaved in a similar manner to rumen epithelium in that they utilized butyrate most readily and converted a large proportion of it into

For comparison exactly similar experiments were made with liver and kidney tissue from adult male rats. The results (Table 8), which concord with those reported by other workers, show no important differences from those obtained with the corresponding tissues of the sheep (Table 7).

The production of ketone bodies on such a scale by the epithelia of sheep fore-stomachs was rather unexpected, and it was considered desirable to try to confirm this by an *in vivo* experiment. For this

purpose a ewe with a permanent rumen fistula was anaesthetized by intravenous injection of Nembutal and operated upon in the manner described by Masson & Phillipson (1951) (the oesophagus and reticulo-omasal orifice were closed by ligatures and the carotid artery and posterior rumen vein exposed for withdrawal of blood samples). The rumen was emptied and washed clean with warm water. Blood samples were withdrawn for ketone body and volatile acid determination. Portions (3 l.) of each of the liquids indicated in Table 9, warmed to about 40°, were placed in the rumen in the order given in the table. Each was allowed to remain for half an hour, blood samples were then taken and the rumen washed out with warm water before the next insertion. Table 9 records the changes in ketone body and volatile acid levels of the blood in the rumen vein and carotid artery. The experiment was repeated on another sheep; in this case blood samples were taken at 5 min., as well as at 30 min., after the final addition of butyrate.

It may be seen that in each experiment the presence of butyrate in the rumen led to several-fold increases in the level of ketones in the blood. In every case the increase was greater in the blood draining the rumen than in the arterial blood, as would be expected if ketone bodies were produced in the rumen wall. A smaller rise of blood ketone bodies occurred with acetate in the rumen, whilst propionate produced no increase in the first experiment and a pronounced decrease in the second. In the first experiment each of the four solutions, after withdrawal from the rumen, was also analysed for ketone bodies. A total of 44·5 and 22·2 mg. respectively (as β-hydroxybutyric acid) was found in the butyrate solutions and 9·0 mg. in the propionate solution. No ketone bodies appeared in any of the other solutions.

DISCUSSION

The marked ability of the epithelia of sheep stomachs to metabolize the lower fatty acids, shown in these experiments, substantiates the conclusions drawn from previous work that an appreciable portion of the butyric acid absorbed is removed before it can enter the circulation. The results also show that this may apply, though to a lesser extent, to propionic and acetic acids. Although too much reliance cannot be placed on the quantitative significance of such *in vitro* studies, it is perhaps of interest to compute that the mean value for butyrate loss in Table 3 would represent a utilization rate of about 0·3–0·5 g. of butyric acid/hr. by the epithelium of the whole rumen, and correspondingly more than this when the reticulum and omasum are also taken into account. The concentration of butyrate used in these experiments is of the order of that normally found in rumen liquor. These epithelia are not glandular and hence would not require the energy of oxidation of the fatty acids for secretory purposes. It is possible, however, that such energy may be utilized in connexion with 'active absorption' of substances from the rumen.

The fact that such a large proportion of the butyric acid was converted to ketone bodies by these tissues is also of considerable interest. Ketone body production on an important scale is generally acknowledged to be confined to the liver in the common laboratory animals. In the experiments reported in this paper, the epithelia of the forestomachs of the sheep were about as active, weight for weight, as liver tissue in the production of ketone bodies. The total wet weight of these epithelia was determined in one animal and found to be over half the weight of the liver, hence these tissues must be considered as important sources of ketone bodies in the sheep. It is possible that such a supplementary production of ketone bodies, which are known to be utilized by other tissues (Breusch, 1948), assists the animal in dealing with the large fatty acid intake from these organs. However, it remains to be seen whether such speculations are valid under normal conditions where mixtures of fatty acids are absorbed from the rumen.

On comparing the activities of all the tissues studied it is of interest to note that only the tissues which converted a large proportion of the butyrate into ketone bodies showed a marked preference for this fatty acid over the other two. The inability of the sheep-brain tissue to metabolize fatty acids is in accordance with the failure of other workers to observe the oxidation of acetate by brain tissue of other animals and of Quastel & Wheatley (1933) to observe the oxidation of butyrate. It appears that brain tissue may be unique in this respect.

The suggestion of McClymont (1951) that enzymes for metabolizing propionic acid in the sheep are concentrated in the liver is not borne out here. McClymont quotes, in support, the observation of Grafflin (1948) that a cyclophorase preparation from rabbit kidney was incapable of oxidizing propionic acid although acetic acid was readily oxidized; both acids were readily oxidized by the liver preparation. In the present experiments propionate was removed most rapidly by kidney and was utilized by all the tissues with the exception of brain and possibly of cardiac muscle. The fate of the propionate is an important question which is being further studied. As mentioned previously, it seems highly probable, from present knowledge of ruminant digestion, that propionate may be the main source of carbohydrate for these animals. It has been demonstrated (Hitchcock & Phillipson, quoted by Phillipson, 1947*b*) that the administration of propionate to the phlorrhizinized sheep leads to the excretion of an

equivalent amount of glucose in the urine. It will be of interest to see whether the direct conversion of propionate to carbohydrate by sheep tissues can be demonstrated *in vitro* and, if so, whether this process is confined to the liver. The observation that the presence of carbon dioxide enhances the utilization of propionate points to a possible fixation of carbon dioxide in the metabolism of the latter. Carboxylation to succinate followed by carbohydrate synthesis would seem an obvious possibility, although there is no good evidence that propionate can be converted directly to succinate in living tissues.

An important difference between the experimental conditions here used and the conditions *in situ* is that in the latter case the tissues usually have available a mixture of the fatty acids. One possibility then is that propionate could be oxidized simultaneously with the other fatty acids and help to promote complete oxidation of the latter instead of ketone body formation. Quastel & Wheatley (1933) found that propionate inhibited ketone body formation from butyrate by liver slices, although their results could be explained simply by selective utilization of propionate. Jarrett & Potter (1950) in the paper already mentioned suggest that propionate, by conversion to pyruvate, may facilitate the metabolism of acetate. The modern concept of antiketogenesis, namely, the provision of pyruvate, which, by carboxylation to oxaloacetate increases the operation of the Krebs cycle can only be applied to tissues with β-carboxylase activity (Baldwin, 1949). It will be of interest to see whether antiketogenesis can be demonstrated in the rumen epithelium.

SUMMARY

1. The ability of rumen epithelial tissue of the sheep to metabolize butyric acid and, to a lesser extent, acetic and propionic acids, under aerobic conditions has been demonstrated; these compounds are amongst the chief products of fermentation in the rumen.

2. A large proportion of the butyric acid was converted to ketone bodies, mainly acetoacetic acid, by this tissue. Ketone bodies were formed also from acetic acid, but not from propionic acid.

3. The utilization of propionic acid was more rapid under an atmosphere containing 5 % of carbon dioxide than under an atmosphere of oxygen.

4. The utilization of the volatile fatty acids by other sheep tissues has also been studied. Ketone bodies were produced from butyric acid by the epithelium of the reticulum and of the omasum as well as by liver tissue; all these tissues metabolized butyric acid more rapidly than acetic or propionic acids. The epithelia of the abomasum and caecum, and kidney tissue, did not show this preference for butyrate and also produced relatively less ketone bodies.

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Chemical Composition of a Sample of Mycelium of *Penicillium griseofulvum* Dierckx

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Both fungal and bacterial processes occur in the formation of soil organic matter (Waksman, 1938), which is itself composed, at least in part, of living micro-organisms and those parts of their remains which are resistant to chemical and enzymic decomposition. A knowledge of the composition of micro-organisms should, therefore, help to shed some light on the constitution of soil organic matter. Whilst much work has been done on the isolation and characterization of low molecular weight constituents of fungi, there are no analyses of mycelium in which the various macromolecular compounds are well defined.

A variety of carbohydrate preparations have been isolated from fungal tissue (Norman, Peterson & Houtz, 1932), and it has long been recognized that the cell wall of many fungal species is chitinous (Norman & Peterson, 1932; Brian, 1949). Protein concentrates have been extracted by Gorgica, Peterson & Steenback (1934) and Bohonos, Woolley & Peterson (1942). Takata (1929), and Akasi (1939) isolated nucleic acid preparations. Claims have been made for the isolation of 'crude lignin' or 'lignin-like complexes' by Thom & Phillips (1932), Phillips (1938) and Pinck & Allison (1944). Porges (1932) and Schulz (1937) studied the effect of cultural conditions on mycelial composition but, for the most part, their fractions are quite arbitrary and sometimes misleading. The literature is summarized and discussed by Foster (1949).

In this paper various analytical methods have been studied from the point of view of their usefulness in the determination of the chemical components, particularly the macromolecular ones, of a sample of mycelium of *Penicillium griseofulvum* Dierckx, grown as a surface pad. This is not a normal or important components of the soil flora, but was chosen for this work, which will be used as a model for future investigations on the predominant component of the soil, because it was readily available.

No precautions were taken to separate spores, or to avoid autolysis, and values given are by no means claimed to be typical of mould mycelium.

MATERIALS AND GENERAL METHODS

Culture of mycelium. I am indebted to Mr P. J. Curtis of the Butterwick Research Laboratories of Imperial Chemical Industries for the following information. Culture: *P. griseofulvum* Dierckx (BRL 374) (ex Prof. H. Raistrick no. P 38). Medium: Czapek-Dox (Raistrick), containing 7·5% crude glucose and 0·1% peptone. The mould was grown in earthenware culture vessels of the type described by Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey (1941) using 1 l. medium in each and an incubation period of 14 days.

Preparation of mycelium. The mycelium, received a few hours after harvesting, was spread thinly on sheets of paper, and by the following day most of the moisture had evaporated, but although allowed to dry at room temperature for over a month, it never became sufficiently crisp to powder satisfactorily in a small C. and N. (Christy & Norris, Chelmsford) laboratory mill (0·5 mm. sieve). However, there was no difficulty with mycelium that was dried thus for only 3 or 4 days, if placed in the hot room at 40° for several hours before milling.

A portion of the powdered mycelium (*M*) was exhaustively extracted in a Soxhlet apparatus with light petroleum (b.p. 40–60°), ether, ethanol, acetone and chloroform, in that order, and again dried in air (*Ms*). Subsequently it was found that acetone, ethanol and chloroform alone were as effective and light petroleum or ether extracted no more material.

A portion of *Ms* was extracted twice with cold water (10 vol. for 30 min. with stirring) and then three times with the beaker standing in a boiling-water bath. The residual mycelium was exhaustively extracted with ethanol and air-dried (*Msw*).

Dry matter. Weighed samples were dried at 100–105° overnight and reweighed.

Total nitrogen. This was determined by a micro-Kjeldahl method using SeO_2:$CuSO_4.5H_2O$:K_2SO_4::1::8 as catalyst.

NH_3 *nitrogen.* This was estimated by microdiffusion in a Conway dish followed by nesslerization (Conway, 1947).

α-Amino-acid N was determined by the ninhydrin-titration method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2·5 and a reaction time of 8 min.

Total P. This was estimated by a modification of the method of Kuttner & Lichtenstein (1932).

Carbohydrate. This was determined by the colour given on heating with orcinol in H_2SO_4 using a glucose standard (Pirie, 1936). The intensity of colour was measured with a photoelectric colorimeter (the Evans Electroselenium Portable model, using Ilford filter 622) and the sugar concn. was 15–100 μg. in a final volume of 10 ml. Equal weights of hexoses give values of 100%, of polyhexoses, 110% (cf. Holden, Pirie & Tracey, 1950). No interference by protein (gelatin), glucosamine hydrochloride, or mannitol was detectable when 50 μg. of these substances were added to 50 μg. of glucose.

Reducing substances. These were determined by Nelson's

Table 1. *Composition of mycelium preparations (moisture free)*

Mycelium	Yield (mg./g. *M*)	Composition (mg./g. mycelium) Ash	N	P	Carbohydrate (as polyhexose)
M—whole mycelium	—	38	31·5	7·0	510
Ms—extracted by organic solvents	700	34	35	7·6	640
Msw—extracted by organic solvents and by water	630	14	34·5	3·3	700

(1944) adaption of the Somogyi method, without the preliminary precipitation of protein.

Amino sugar estimations. These were made on hydrolysates freed from the HCl used by repeated distillation *in vacuo*. The method of Elson & Morgan (1933) was modified by Mr M. V. Tracey of this department according to Blix (1948) and Immers & Vasseur (1950). Amino sugar was also determined by distillation with saturated Na_3PO_4 solution plus sodium borate, and estimation of the NH_3 produced by nesslerization (Tracey, 1951).

Paper chromatography. One-dimensional, descending paper chromatography was used for qualitative and quantitative examination of the carbohydrate components (Flood, Hirst & Jones, 1948). The sugar spots were developed with either ammoniacal $AgNO_3$ (Partridge, 1948) or aniline phthalate in glacial acetic acid (Hough, Jones & Wadman, 1950).

Colloidal material. This was isolated by dialysis in a cellophan membrane into frequently changed distilled water.

EXPERIMENTS AND RESULTS

In general, estimations were carried out, not only on whole mycelium (*M*), but also on mycelium that had been exhaustively extracted with organic solvents (*Ms*) and then with cold and hot water followed by a further extraction with ethanol (*Msw*) (cf. Table 1). Whether or no extracted material interfered with the determinations could be recognized.

Organic solvent extraction. This removed 31% of the dry weight of *M*. In the preparation of *Msw*, further extraction with ethanol removed another 1% of the dry weight of *M*. A little more fat-soluble material was liberated by hydrolysis of *Msw* with N-HCl in a boiling-water bath for 2 hr. Amounting to 2·5% of the dry weight of *M*, it was extracted from the solid residue by boiling ether-ethanol mixture (1:1) and from the aqueous layer by ether. More severe conditions of hydrolysis or refluxing over a flame produce traces of black 'humin' material which is itself soluble in ethanol. A total of 34·5% of the dry weight of *M* is, therefore, actually or potentially extractable from the mycelium with organic solvents.

No attempt was made to prepare pure compounds from these extracts, with the exception that mannitol was readily isolated by extraction with water, and after recrystallization had m.p. 165°, unchanged when mixed with an authentic specimen. The hexaacetate had m.p. 120°, also unchanged on admixture with an authentic specimen.

The combined material extracted with organic solvents contained 22% of the total N of mycelium *M*, and 24% of the total P.

Water extraction (cf. Tables 2*a* and *b*). Mycelium *M* (1 g.) extracted four times with water (25 ml.) in a steam bath, lost 29% of its dry weight. On dialysis 15% of the dry matter of the extract was retained as colloidal material. The extraction of mycelium *Ms* yielded dry matter equivalent to only 5·5% of the dry weight of mycelium *M*. On dialysis, 50% of the dry matter of the extract was retained.

Table 2*a*. *Composition of water extract of mycelium (M)*

	Content mg./g. dry matter	Percentage of total constituent in *M*	Percentage of each constituent in colloidal fraction
Nitrogen	35	32	19
Carbohydrate (as glucose)	220	10	36
Reducing substance (as glucose)	20	—	—

Table 2*b*. *Composition of water extract of solvent-extracted mycelium (Ms)*

	Content mg./g. dry matter	Percentage of total constituent in *M*	Percentage of each constituent in colloidal fraction
Nitrogen	40	7	75
Phosphorus	55	45	20
Carbohydrate (as glucose)	330	3	100

Table 3. *α-Amino-acid* N *content of* HCl *hydrolysates of mycelium*

(Mycelium taken into 12 N-HCl solution, was diluted with an equal volume of water, and heated in a sealed tube immersed in boiling water, for 20 hr. The HCl was removed by vacuum distillation.)

Mycelium	α-Amino-acid N (mg./g. N of mycelium)	α-Amino-acid N (mg./g. N of mycelium *M*)
M—whole mycelium	46	46
Ms—extracted by organic solvents	56	44
Msw—extracted by organic solvents and by water	58	40

Protein

Results are summarized in Table 3. A protein concentrate which was free, as far as could be ascertained, from other nitrogenous material was prepared by extraction of *Ms* with cold 2 N-NaOH, precipitation of the carbohydrate with an equal volume of ethanol and, after bringing to pH 7 with glacial acetic acid, removal of the bulk of the ethanol by

distillation *in vacuo*. The small amount of insoluble material was spun off and contained less than 2% N. Trichloroacetic acid solution (20%) was added to the filtrate to a final concentration of 5% and the precipitate centrifuged off and washed with ethanol. About 0·04 g. of a pale-brown powder containing 9% N was thus isolated from 1 g. of *Ms*. After acid hydrolysis 720 mg. α-amino-acid N/g. total N was found. The apparent amino-sugar content, determined by the Elson & Morgan method was less than 1%, while in the case of Tracey's (1951) method, the NH_3-N (50 mg./g. total N) was identical with the N given off as ammonia on distillation with saturated sodium phosphate solution. How far this figure of 720 mg. α-amino-acid N/g. total protein N is representative of the mycelial protein as a whole is not known, but under the conditions of hydrolysis used it is fairly typical of other protein preparations examined.

Assuming, therefore, that the mycelial protein of *M* yields 440 mg. α-amino-acid N/g. total N, and this value represents 72% only of the total protein N, then about 60% of the total N of mycelium *M* must be present in protein, which, using the conventional conversion factor 6·25, corresponds to 12% of protein in mycelium *M*.

Nucleic acid

An estimation of nucleic acid N and P was made following the method of Schneider (1945). Trichloroacetic acid solution (12·5 ml. of 10%) was added to mycelium *Ms* or *Msw* (1 g.) which had been allowed to soak in water (5 ml.) at room temperature for 30 min., and the mixture stirred for 20 min. After filtering with suction through madapollam the residue was refluxed, first with ethanol, then with chloroform. The air-dried product was then extracted with 5% trichloroacetic acid (25 ml.), with the extraction tube standing in boiling water, for 15 min. After filtering and washing, first with 5% trichloroacetic acid and then with water, extract and washings were combined. The cold trichloroacetic acid removed little more N and P from *Ms* than did water, and the extract from *Msw* was very slight. With hot trichloroacetic acid the amount of P extracted amounted to 110 mg./g. of total P in *M*, and of N to 70–100 mg./g. of the total N in *M*. Accepting the values found for phosphorus extracted, and assuming that nucleic acid contains about 10% P, this leads to a value of 8·0 mg. of nucleic acid/g. mycelium *M*.

After hydrolysis of *Ms* with N-H_2SO_4 for 7 hr. in a sealed tube immersed in boiling water, ribose was detected in the hydrolysate by paper chromatography. Quantitative measurements using a known amount of xylose as standard showed the presence of about 1·0 mg. ribose/g. *M*.

Chitin

The presence of polyacetylglucosamine in the mycelium (10 g.) was shown by hydrolysis with 6N-HCl at 100° for 6 hr. of the fraction of the mycelium remaining after extraction with 2N-NaOH. Following distillation of the acid *in vacuo*, glucosamine hydrochloride was isolated from the solid residue by rubbing with acetone, followed by two crystallizations from aqueous ethanol. The colourless crystals (0·3 g.) had $[\alpha]_D^{20°} + 71°$ (*c*, 1·5 in water), after standing. An authentic sample of glucosamine hydrochloride gave $[\alpha]_D^{20°} + 72°$ (*c*, 1·5 in water), after standing. (Found N, 6·6. $C_6H_{14}O_5NCl$ requires N, 6·5%.) The osazone was identical in crystalline form with glucosazone prepared from an authentic specimen of glucosamine hydrochloride, and had m.p. and mixed m.p. 204–205°.

A mixture of the mycelium (10 g.) with 72% (w/w) H_2SO_4 (20 ml.) after standing for 2 days was diluted to 700 ml. and refluxed for 10 hr. The acid was partially neutralized with $Mg(OH)_2$ and the product steam distilled. A definite positive test for acetic acid with lanthanum chloride and iodine (Krüger & Tschirch, 1930) was not given by this distillate, but when volatile reducing substances were fixed by oxidation with $KMnO_4$ beforehand, the steam distillate then gave a positive test. This effect is presumed to be due to the sulphur dioxide present in the hydrolysate, since sulphites were shown to reduce the sensitivity of the colour reaction. A small amount of a colourless, crystalline, water-insoluble material, which has not been identified, was filtered from the main bulk of the distillate which, after neutralization with NaOH was evaporated to small volume, acidified with HCl and extracted with ether. After evaporation of the ether, the extract smelt strongly of acetic acid and gave a *p*-phenylphenacyl ester, which after recrystallization had m.p. 109°. An authentic sample prepared from acetic acid had m.p. 110° and the mixed m.p. was 109°.

Adapting the method used by Black & Schwartz (1950) for the estimation of chitin in crawfish waste, mycelium (1 g.) was extracted once with cold 5% (w/v) NaOH (25 ml.) by standing, with frequent stirring, for 30 min. The insoluble material, spun down or filtered through madapollam under suction was extracted three times with the beaker standing in boiling water. Further extraction removed only traces of N. The residue was washed with water, N-acetic acid, again with water, refluxed with ethanol, followed by acetone, then chloroform, and air dried. Table 4 gives yields and analyses of the product, *M.alk.* The cold alkaline extract was used as a source of alkali-soluble carbohydrate.

After hydrolysis of *M.alk.* or chitin itself with 6N-HCl for 16 hr., no α-amino-acid N was found. Assuming, therefore, that the N content of *M.alk.* is entirely derived from amino-sugar residues, a minimum of about 9% of the total N of mycelium *M* is present in this form. This corresponds to about 19% of chitin in *M.alk.* and to about 4% in mycelium *M*.

Hydrolysis of mycelium with HCl, removal of the acid by distillation *in vacuo*, and estimation of amino sugar, gave further information. Initial experiments were carried out on an authentic sample of chitin (100 mg.) brought into 12N-HCl solution (1 ml.) by standing at room temperature, then

Table 4. *Alkali-resistant residue* M.alk.

Mycelium source	Yield (mg./g. of mycelium source)	Yield (mg./g. of mycelium *M*)	Nitrogen content (mg. N/g. *M.alk.*)	Nitrogen content (mg. N in *M.alk.*/g. N in mycelium *M*)
M—whole mycelium	230	230	13	84
Ms—extracted by organic solvents	320	220	13	90

The above figures refer to moisture-free material

diluting and heating in a sealed tube in boiling water. Amino-sugar content was estimated by Tracey's (1951) method. Heating with 6N-HCl for 3–15 hr. gave values between 86 and 92% of the total N given off as NH_3, without a distinct maximum; 4N-HCl for 10 hr. gave about 75% of the total N as NH_3, and 3N-HCl for 10 hr. gave about 70%. Irving (1909), on polarimetric evidence, claimed that chitin was completely hydrolysed by conc. HCl in 8–10 hr. at 40–45°. However, chitin hydrolysed with 10N- or 12N-HCl for 10 hr. at 37° gave only 40% of its N as NH_3 when distilled with sodium phosphate. Even after 48 hr. hydrolysis, no more than 50% was obtained.

On the basis of these results, hydrolysis of mycelium was carried out by taking up the sample (100 mg.) in 12N-HCl (1 ml.), diluting after 2–3 days (when the mycelium was almost completely dispersed) with an equal volume of water and heating for 6 hr. in a sealed tube in boiling water. A small amount of black, insoluble material was formed, and care was taken to carry out estimations by the Elson & Morgan method on the clear, pale yellow or brown supernatant liquid.

In the case of mycelium hydrolysates the N volatile on distillation with sodium phosphate solution includes a proportion of NH_3-N derived from the degradation of amino-acids and this must be allowed for. However (Table 5) about 20% of chitin N is also degraded to NH_3 and hence the net amounts of N in column 3 are taken to represent only 80% of the total chitin N. A correction is made for this in column 4. About 12% of the total N of mycelium *M* occurs in amino-sugar residues, corresponding to 5·5% chitin in the mycelium.

Discordant results are obtained unless the laboratory atmosphere is clear. The size of the blanks are reduced if the steam source is made alkaline. Under these conditions the following results were obtained:

N-Acetylglucosamine. (Found: CH_3CO, 19·3. Calc. for $C_6H_{12}O_5N.COCH_3$: CH_3CO, 19·4%.)

A chitin sample. (Found: N, 6·0; CH_3CO, 19·2. Calc. for anhydroacetylglucosamine, $C_6H_{10}O_4N.COCH_3$: N, 6·9; CH_3CO, 21·2%.)

M.alk. (Found: CH_3CO, 4·1. Calc. for a chitin content of 19%: CH_3CO, 4·0%.)

Ms. (Found: CH_3CO, 1·7, corresponding to 1·2% CH_3CO in mycelium *M*.)

Msw. (Found: CH_3CO, 1·75, corresponding to 1·1% CH_3CO in mycelium *M*.)

M. (Found: CH_3CO, 1·5%.)

If mycelium *M* contains 5·5% of chitin, it would have 1·2% CH_3CO. The higher percentage found in *M* itself must be due to the presence of acetyl compounds or other materials giving rise to volatile acid on hydrolysis, among the fat-soluble components. No higher fatty acids than acetic were detected in the distillate by paper chromatography using the method of Brown (1950). The titration figure was not reduced by the addition of $KMnO_4$ before distillation, confirming the absence of formic acid.

Carbohydrate

To estimate the carbohydrate content of mycelium by the orcinol method (Pirie, 1936), which obviates interference from protein or amino-sugar, the sample is taken up in 72% (w/w) H_2SO_4 (4–6 mg./ml.) by allowing to stand at room temperature for 1–3 days, when the solution from fat-free samples is completely clear and yellow to brown in colour. Mycelium *M* gives an opalescent solution due to acid-insoluble fatty components. The estimation is carried out at a suitable dilution. The values obtained were unaffected by the time the mycelium was allowed to stand in acid, up to 7 days.

The polyhexose content of *M* is taken as 45%, calculated from the value obtained for *Ms* (Table 1). A certain amount of orcinol-positive material is therefore extracted by organic solvents, and this is presumed to be identical with the dialysable carbohydrate extracted by water from *M* (Table 2*a*). Although this water extract contains a small amount of reducing substance, no reducing sugar was

Table 5. *Glucosamine* N *in hydrolysates of mycelium*

(Hydrolysis by 6N-HCl for 6 hr. Results as mg./g. total N in material except in last two columns.)

Material	Ammonia N (*AN*)	Distilled N (*DN*)	*DN* – *AN*	(*DN* – *AN*) ×1·25	N (E & M)	Total N in mycelium *M* (mg./g.) (*DN* – *AN*) ×1·25	Total N in mycelium *M* (mg./g.) N (E & M)
Glucosamine HCl	50–60	960–1050	900–1000	—	96–106	—	—
Chitin	70–80	870–920	780–850	—	93–110	—	—
Mycelium *M*	80–90	180–190	90–110	120	90–110	120	100
Mycelium *Ms*	60–70	190	120–130	150	130–140	117	100
Mycelium *Msw*	50–70	190–220	140	175	180	122	125
M.alk.	30–60	800–850	790–820	1000	720–870	—	—

N (E & M) refers to glucosamine N estimated by Elson & Morgan's (1933) method.
Distilled N refers to glucosamine N estimated by distillation with saturated sodium phosphate (Tracey, 1951).

Estimations of acetyl content were made by allowing the mycelium (50 mg.) to stand for several days in 72% (w/w) H_2SO_4 (0·5 ml.), diluting to 12·5 ml. (to give an approximately N-acid solution) and heating in a boiling-water bath for 3 hr. After unsealing, the tubes were allowed to stand uncorked for about an hour to free the solution from volatile inorganic acid. No acetic acid is lost. 1 ml. of the solution, mixed with about 4 g. $MgSO_4.7H_2O$ (to accelerate the distillation of the acid; Olmsted, Whitaker & Duden, 1929), was distilled in a Markham (1942) still at the rate of about 1 drop/sec., until exactly 10 ml. of distillate had been collected. The distillates were titrated with 0·2N-$Ba(OH)_2$ solution from a Conway microburette. Blank distillations were made from 1 ml. N-H_2SO_4 and 4 g. $MgSO_4.7H_2O$.

detected in it or the ethanolic extract by paper chromatography. After hydrolysis of a sample of ethanolic extract with N-H_2SO_4 glucose was detected by paper chromatography.

Only a small amount of colloidal carbohydrate is extracted by water from *M* or *Ms* (Tables 2*a* and *b*), but larger amounts are dispersed by cold 2N-alkali. By precipitation of the alkaline solution with an equal volume of ethanol (Norman & Peterson, 1932) a predominantly carbohydrate material is obtained representing 10–15% by weight of *M* and containing only 0·6–1·0% N. On hydrolysis with N-H_2SO_4 for 10 hr. in a sealed tube in boiling water, it was not taken entirely into solution, which contained only 600 mg. of reducing sugar (as glucose)/g. polysaccharide. On hydrolysis for 20 hr., after preliminary solution in 72% (w/w) H_2SO_4, 850 mg. reducing sugar/g. of polysaccharide were obtained.

The alkali-extracted mycelium, *M.alk.*, contains about 74% of carbohydrate (as polyhexose), associated with chitin.

Ms, taken up in 72% (w/w) H_2SO_4 by allowing to stand for 2 days, diluted to give an N-acid solution, and hydrolysed for 20 hr. gave 73% of its dry weight as reducing substance, calculated as glucose. Paper chromatography indicated the presence of glucose, galactose, and mannose, approximately in the ratio 10:1·7:1, and these three sugars were also present in *M.alk.*, whereas the alkali-soluble polysaccharide contained only glucose. An osazone was prepared from a hydrochloric acid hydrolysate of this material (after removal of acidic and basic compounds with resin columns), identical in crystalline form, melting point and mixed melting point with an authentic specimen of glucosazone. Under conditions which readily yielded cellobiose octaacetate from cotton-wool cellulose, no such compound could be isolated from this alkali-soluble glucose polymer.

The presence of galactose in the mycelium was confirmed by oxidation with HNO_3 of an HCl hydrolysate of *Ms* (3 g.) freed from acidic and basic material, and isolation of mucic acid, m.p. 211–212° (decomp.); mixed melting point with an authentic specimen 212–213° (decomp.).

DISCUSSION

The preceding results lead to the figures for the composition of the mycelium given in Table 6. There is no indication that the mycelium contains 'lignin-like substances', and it is quite erroneous to suppose (as did Pinck & Allison, 1944) that the mycelium fraction insoluble after standing for 2 hr. in 72% (w/w) H_2SO_4 followed by refluxing after dilution to 3%, can be characterized as crude lignin. Even *M.alk.*, which certainly after alkali extraction contains no lignin, requires at least 24 hr. to disperse completely. On dilution of the clear solution and refluxing, a small amount of black material is formed but this is an invariable artifact in the hydrolysis of material containing protein or amino sugar and carbohydrate.

Table 6. *Composition of mycelium M (moisture-free)*

(The air-dried mycelium contains in addition, about 10% moisture.)

	mg./g. *M*
Fat and organic solvent-soluble material	345
Residual, dialysable, water-soluble material	30
Polyhexose carbohydrate	450
Protein	120
Chitin	55
Nucleic acid	10
Residual, unextracted ash	10
Total	1020 mg.

Nitrogen is distributed among the organic solvent extract (22%), residual water-extractable and dialysable material (5%), protein (60%), chitin (12%), and nucleic acid (4%).

About 70% of the total phosphorus is extracted by organic solvents and water and 10% remains in *M.alk.* (which surprisingly contains 0·3% P). Nearly 10% is present in nucleic acid.

The composition of *M.alk.* approximates to: polyhexose 74%; chitin 19%; ash, 1%. The remainder has not been accounted for.

SUMMARY

1. The composition of a sample of mycelium of *Penicillium griseofulvum* has been studied with particular reference to the high molecular weight components.

2. Fats and compounds soluble in organic solvents amount to about 35%.

3. Protein (about 12%) was estimated by a determination of α-amino-acid nitrogen on various fractions hydrolysed with hydrochloric acid.

4. Carbohydrate (about 50%) occurs for the most part as polyhexose. Glucose is the predominant sugar residue, but galactose and probably mannose are also present in the polysaccharide.

5. The presence of chitin (about 5·5%) was confirmed, but it was only isolated associated with carbohydrate.

6. There is no indication of the presence of lignin.

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The Component Acids of the Fats of some Indian Fresh-water Fish

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The fats studied were from the body, viscera and liver of bhakur fish (*Catla buchanani*) and from the liver of pahuna fish (*Wallago attu*). These fish were from the River Ganges at Benares.

METHODS

The separated tissues (body, viscera or liver) were cut up into small pieces and extracted exhaustively with hot acetone. The extracted material was then taken up in light petroleum (b.p. 40–60°), the watery portion separated and the lipids recovered and dried at 100° under reduced pressure. The body and liver tissues were also treated by alkali digestion as recommended by Rapson, Schwartz & Van Rensburg (1943), but little fat could be extracted by this treatment. The fats were kept in 10 vol. of acetone at 0° to separate the phosphatides, if present, from the glycerides.

The fats were hydrolysed, and the mixed fatty acids recovered were resolved into groups of simpler mixtures of acids by the lithium salt-acetone (Tsujimoto, 1920; Tsujimoto & Kimura, 1923) and lead salt-ethanol methods. Each group of the acids was separately converted into methyl esters, taking precautions recommended by Bjarnason & Meara (1944), and fractionated through an electrically heated and packed column. From the iodine values and equivalents of the ester fractions the composition of the latter were calculated by the method of Hilditch (1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturation, e.g. −2·0 (monoethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each group from which the mean equivalent of each of the homologous ester groups (C_{16}, C_{18}, C_{20}, C_{22} and C_{24}) follows. From the composition of each of the separated groups of fatty acids, that of the original fat was obtained.

RESULTS

The lipids and phosphatide contents of the tissues and the iodine values of the fats are given in Table 1.

Bhakur fats. The mixed fatty acids obtained by the hydrolysis of fats, as described earlier, from body (159·6 g.), viscera (202·8 g.) and liver (29·3 g.) were

Table 1. *Components of tissues of bhakur and pahuna*

	Bhakur			Pahuna
	Body	Viscera	Liver	liver
Water content (% on wet tissues)	67·5	40·2	72·7	68·3
Lipid content (% on wet tissues)	6·3	44·8	8·5	5·1
Lipid content (% on dry tissues)	19·0	75·0	31·3	16·1
Phosphatides in lipids (%)	0	0	5·4	12·5
Iodine value of the fat	97·9	87·2	90·5	122·9

Table 2. *Fractionation of tissue fats of bhakur*

		Body		Viscera		Liver	
	Group description	Weight (%)	Iodine value	Weight (%)	Iodine value	Weight (%)	Iodine value
A	Lead salt ethanol-insoluble	31·8	1·4	36·8	5·3	32·8	22·4
B	Lithium salt acetone-insoluble but lead salt ethanol-soluble	47·8	111·8	49·9	105·0	67·2	138·0
C	Lithium salt acetone-soluble	20·4	248·2	13·3	237·6	—	—

Table 3. *Fractionatoin of methyl esters of bhakur body-fat acids A, B and C*

Fraction	Weight (g.)	Saponification equivalent	Iodine value
	Methyl esters of acids *A*		
*A*1	3·05	254·6	0·2
*A*2	3·29	265·7	0·4
*A*3	3·86	268·0	0·6
*A*4	3·93	270·7	0·6
*A*5	4·01	271·2	0·6
*A*6	4·80	271·5	1·1
*A*7	3·26	272·2	1·1
*A*8	3·67	274·8	1·6
*A*9	5·37	284·3	2·4
*A*10	4·24	289·2*	11·6
	39·48		
	Methyl esters of acids *B*		
*B*1	2·38	253·7	51·6
*B*2	3·16	267·1	72·0
*B*3	4·53	270·0	83·1
*B*4	4·97	272·7	83·8
*B*5	5·22	273·3	90·1
*B*6	3·57	273·8	93·0
*B*7	4·65	277·6	93·6
*B*8	4·75	278·0	99·9
*B*9	2·33	278·5	100·2
*B*10	5·02	278·7	102·6
*B*11	3·68	279·4	107·4
*B*12	4·20	281·9	112·3
*B*13	4·46	285·9	134·8
*B*14	2·04	299·6	169·7
*B*15	4·25	327·3*	183·1
	59·66		
	Methyl esters of acids *C*		
*C*1	1·38	271·5	128·5
*C*2	2·84	281·2	153·3
*C*3	2·66	299·3	176·0
*C*4	3·67	308·4	256·7
*C*5	2·95	325·5	292·0
*C*6	3·05	335·6	332·7
*C*7	5·38	344·8	336·7
*C*8	3·90	345·8	340·9
*C*9	2·98	358·0*	143·6
	28·81		

* Equivalents of esters (freed from unsaponifiable matter): *A*10, 285·5; *B*15, 317·6; *C*9, 350·1.

separated into simpler groups of acids by the methods indicated in Table 2.

Each group of the acids was separately converted into methyl esters, which were fractionally distilled under reduced pressure through the column mentioned. The fractionation data for the methyl esters of the three groups of the body fat are summarized in Table 3.

The percentage composition, calculated from iodine values, and the equivalents and the mean unsaturation determined for each group of acids, and thence the component acids of the body fat, are recorded in Table 4.

Similarly, the component acids of the visceral and liver fats of bhakur were studied and their fatty acid compositions are given Tables 5 and 6, respectively.

Pahuna liver fat. 37·5 g. of the lipid, obtained in the usual manner from 736 g. of wet tissues, deposited 4·7 g. of crude phosphatides from a 10% acetone solution at 0°. As the quantity of the acids available was small it was decided, unlike the bhakur liver fatty acids, to convert them as such into methyl esters and fractionate. The fractionation data are given in Table 7 and the figures for component fatty acids in the whole fat in Table 8.

DISCUSSION

Bhakur body, viscera and liver fats. It is unfortunate that not much work has so far been done on the fats of fresh-water fish, specially on those of Indian origin. Analyses of fats from different parts of the same animal have rarely been recorded, but such data as are available indicate that significant differences in composition may occur. This view is confirmed by the present studies.

In Tables 4–6 are given the component acids of bhakur body, viscera and liver fats. The saturated acid contents of body, viscera and liver fats are about 38, 37 and 30%, respectively, and the contents of

the individual acids are somewhat similar—the chief difference being the much lower content of palmitic acid in the liver fat. It is also interesting to note the gradual increase in the relative proportions of saturated acids of higher molecular weights from body to viscera and to liver fats. The differences (25 %), of viscera C_{18} (37 %) and of liver C_{20} (26 %), although the C_{18} acid content (25 %) of the liver fat is also almost equally high. Unsaturated C_{14} and C_{24} acids are minor components of these fats. In the visceral and the liver fats the unsaturated C_{16} acid group is much lower than in the body fat, but these

Table 4. *Component acids of bhakur body fat*

Acids	*A* (31·8 %)	*B* (47·8 %)	*C* (20·4 %)	Total	Whole fat excluding unsaponifiable (%, w/w)	Moles %
Myristic	1·8	1·1	—	2·9	2·9	3·5
Palmitic	24·0	4·9	—	28·9	29·0	30·6
Stearic	5·2	1·3	—	6·5	6·5	6·2
Unsaturated:						
C_{14}	Trace	0·2	—	0·2	0·2	0·2
C_{16}	0·4	23·2	1·7	25·3	25·3	26·9
C_{18}	0·4	13·7	3·8	17·9	17·9	17·3
C_{20}	—	3·3	4·2	7·5	7·5	6·6
C_{22}	—	—	10·1	10·1	10·1	8·3
C_{24}	—	—	0·5	0·5	0·6	0·4
Unsaponifiable	Trace	0·1	0·1	0·2	—	—
Mean unsaturation of:						
C_{14}	−2·0	−2·0	−2·0	−2·0	—	—
C_{16}	−2·0	−2·0	−4·0	−2·7	—	—
C_{18}	−2·0	−2·3	−5·0	−2·5	—	—
C_{20}	−2·0	−5·0	−8·0	−5·7	—	—
C_{22}	—	—	−9·0	−9·0	—	—

Table 5. *Component acids in groups A, B, C and in the whole bhakur visceral fat*

Acids	Component acids in groups: *A* (36·8 %)	*B* (49·9 %)	*C* (13·3 %)	Component acids in whole fat (%, w/w)	Moles %
Myristic	1·3	1·5	—	2·8	3·4
Palmitic	24·7	0·6	—	25·3	26·9
Stearic	7·4	0·5	—	7·9	7·6
Arachidic	1·4	—	—	1·4	1·2
Unsaturated:					
C_{14}	Trace	0·4	1·0	1·4	1·6
C_{16}	0·7	6·0	3·3	10·0	10·8
C_{18}	0·7	33·2	3·3	37·2	36·1
C_{20}	0·6	7·7	3·6	11·9	10·6
C_{22}	—	—	2·1	2·1	1·8
Unsaponifiable	Trace	Trace	Trace	—	—
Mean unsaturation of:					
C_{14}	−2·0	−2·0	−2·0	−2·0	—
C_{16}	−2·0	−2·0	−4·0	−2·7	—
C_{18}	−2·0	−2·3	−5·0	−2·5	—
C_{20}	−2·0	−5·0	−8·0	−5·7	—
C_{22}	—	—	−9·0	−9·0	—

become marked in the unsaturated portions. Here also, in a general way, the relative proportions of acids of higher molecular weights increase in the same order accompanied by a general decrease in the mean unsaturation—the mean unsaturation of C_{16} and C_{18} acids in the visceral fat deviating slightly from the rule. In the unsaturated acid portions the chief component acid in the fat of body is C_{16} fats are richer in unsaturated C_{18} acids. Liver fat is rich in C_{20} acids. Visceral fat has a small amount of C_{22} acids, but body and liver fats have larger and almost equal amounts (10 %) of this group.

The analyses show large differences in the contents of the fatty acids in the body, visceral and liver fats of bhakur, specially in the unsaturated acid portions. These results are somewhat different from

those for jacopever (*Sebastichthys capensis*) (Van Rensburg, Rapson & Schwartz, 1945), inasmuch as that the jacopever visceral fat resembles the body fat; but in the liver fat the proportion of C_{18} acids is

differences in the unsaturated acids of the body and liver fats of turbot and halibut. Hilditch & Pathak (1949) found that blubber and liver fats of the common seal showed distinct differences in their

Table 6. *Component acids of bhakur liver fat*

Acids	*A* (32·8%)	*B* (67·2%)	Total	Acids excluding unsaponifiable (%, w/w)	Acids excluding unsaponifiable Moles %
Myristic	0·6	—	0·6	0·6	0·7
Palmitic	16·5	2·4	18·9	19·0	21·3
Stearic	4·1	2·3	6·4	6·4	6·5
Arachidic	3·1	—	3·1	3·1	2·9
Unsaturated:					
C_{14}	Trace	—	Trace	Trace	Trace
C_{16}	1·4	5·5	6·9	7·0	7·9
C_{18}	0·9	24·3	25·2	25·4	25·8
C_{20}	5·8	20·5	26·3	26·5	24·8
C_{22}	—	9·3	9·3	9·4	8·1
C_{24}	—	2·5	2·5	2·6	2·0
Unsaponifiable	0·4	0·4	0·8	—	—
Mean unsaturation of:					
C_{16}	−2·0	−2·0	−2·0	—	—
C_{18}	−2·0	−2·7	−2·7	—	—
C_{20}	−2·0	−5·1	−4·8	—	—
C_{22}	—	−6·5?	−6·5?	—	—
C_{24}	—	−8·0?	−8·0?	—	—

Table 7. *Fractionation data of methyl esters of pahuna liver fat acids*

Fraction no.	Weight (g.)	Saponification equivalent	Iodine value
1	1·82	262·0	27·9
2	2·07	270·3	28·9
3	1·84	278·0	41·1
4	2·07	288·7	79·1
5	2·37	293·6	92·7
6	2·05	295·6	98·9
7	2·37	301·3	120·0
8	2·27	318·6	207·6
9	1·97	333·7	259·0
10	2·10	338·3	283·2
11	2·32	353·7*	104·9

* Equivalent of ester (freed from unsaponifiable matter): 327·0.

Table 8. *Component acids of pahuna liver fat*

Acids		%	(%, w/w)	Moles %
Myristic		1·44	1·5	1·8
Palmitic		14·15	14·2	15·9
Stearic		4·00	4·0	4·1
Unsaturated:	C_{14}	0·42 (−2·0)	0·4	0·5
	C_{16}	8·42 (−2·0)	8·4	9·5
	C_{18}	32·45 (−2·4)	32·6	33·2
	C_{20}	19·76 (−5·3)	19·8	18·5
	C_{22}	19·00 (−8·0)	19·1	16·5
Unsaponifiable		0·36	—	—

about 46 as compared with 28 % for the body whilst the C_{20} and C_{22} acids are 13 and 8 % as compared with 22 and 17 % for the body fat. Other small noticeable differences (of 2–3 %) in the body and the liver fats are in palmitic, stearic and unsaturated C_{24} acids. Lovern (1932, 1937) found similar saturated acids and still greater differences in their unsaturated acids.

Unfortunately not many visceral fats have been studied so far. But some of the analyses of visceral fats have been made in cases in which data is also available for the blubber fat of the same animal, even if it has not been studied by the same person. The composition of herring visceral fat found by Hilditch & Pathak (1948) is widely different from that of herring body fat given by Lovern (1938) with respect to unsaturated acids and there are smaller differences in the saturated acids. The ling visceral and liver fat analyses of Shorland (1939) show specific differences in their C_{18}, C_{20} and C_{22} acids.

Pahuna liver fat. In comparison with bhakur, the pahuna has more phosphatide in the liver lipids, and the liver fat is less rich in saturated acids. Palmitic acid is the chief saturated acid in both. The C_{16} unsaturated acid content is similar. The chief unsaturated components of pahuna liver fat are C_{18} unsaturated acids (33 %, which is higher than in the bhakur), but the C_{20} acid group (20 %) and C_{22} acid group are major components. Comparison with published figures for fats of fresh-water fish from other parts of the world gives the impression that the Indian fish livers are low in C_{16} and high in C_{22} unsaturated acids, but the comparison may not be valid, for the published figures are generally not for liver fats, known to be richer in acids of higher molecular weight.

It is worth while to draw attention to the potential source of fat for industrial purposes in the viscera of

bhakur. The wet tissue, now discarded, contains 45% of fat, the recovery of which should be remunerative.

SUMMARY

1. The compositions of body, viscera and liver lipids from bhakur (*Catla buchanani*), and of liver fat of pahuna (*Wallago attu*) have been studied. Preliminary separations of the fatty acids into groups differing in unsaturation have been carried out with the help of lithium and lead salts in acetone and ethanol, respectively, and the compositions studied by the ester fractionation procedure.

2. The component acids of bhakur body, viscera and liver fats show significant differences in their proportions and degree of unsaturation. The fat content of viscera is remarkably high, 45%, as compared to 6·3% in the body and 8·5% in the liver on wet tissues, and industrial exploitation of the fat has been suggested.

3. The liver lipids of pahuna contain more phosphatides than bhakur liver fat, and there are minor differences in the acid components.

We are glad to express our most cordial thanks to Prof. T. P. Hilditch, F.R.S., for his very useful suggestions and kind help during the progress of this work.

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The Formation of Hydrogen Peroxide by α-Radiation in the Presence of the Enzyme Carboxypeptidase

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The enzyme carboxypeptidase is inactivated by exposure to either α-radiation or X-radiation in aqueous solution. In all except the most concentrated solutions, the inactivation is due almost entirely to energy dissipated as ionization and excitation, in the water, leading to the formation of radicals or other active chemical intermediates which enter into the reaction with the enzyme. Whilst this is true of both types of radiation, the number of enzyme molecules inactivated per unit energy dissipated in the water by α-radiation is only about one-twentieth of the number inactivated by an equal dose of X-radiation. When these experimental observations were reported (Dale, Gray & Meredith, 1949), it was pointed out that the small amount of inactivation brought about by α-radiation could, in fact, be reasonably ascribed to the very low energy electrons, or δ-rays, which branch off as spurs along an α-particle track, in which case it would appear that the main part of the α-ray energy which is dissipated within a narrow column is almost without effect on the enzyme. It was natural to associate the absence of effect on the enzyme with the well established fact that α-ray energy, unlike X-ray energy, gives rise to hydrogen peroxide and hydrogen when dissipated in pure air-free water, and to suppose that at all enzyme concentrations used in our experiments the radicals formed at very high concentration within the α-ray column combine together to form hydrogen peroxide and hydrogen before diffusion has brought them into effective collision with enzyme molecules. In this case, the presence of the enzyme should have little or no effect on the hydrogen peroxide yield, since the enzyme does not react with hydrogen peroxide. An alternative interpretation might be to assume that, owing to the very high concentration of radicals, interaction with enzyme molecules is confined to

those few molecules lying close to the track, so that many radicals in succession give up their energies to a few already inactivated enzyme molecules. The presence of enzyme in this case should cause a decrease in hydrogen peroxide yield. The fact that carboxypeptidase activity is hardly affected by high concentrations of hydrogen peroxide has made possible a comparison of the hydrogen peroxide yield by α-radiation in water and in a solution of carboxypeptidase. The results show that the alternative mechanism has to be rejected.

EXPERIMENTAL

The experimental technique followed closely that of former experiments (Dale *et al.* 1949). Radon was divided approximately equally between, and sealed into, two small and thin glass bulbs. These were then broken within a few minutes of pumping, one in a gas-tight vessel completely filled with water, and one in a similar vessel filled with the carboxypeptidase solution. The amount of radon in each solution was measured by following the rise of the γ-activity of the solutions, using a conventional ionization chamber and electrometer valve circuit. An irradiation of about 1·5 hr. was given, and an attempt made to compensate for a small difference in the distribution of radon between the two glass bulbs by adjusting the time of irradiation. The irradiation was ended by pouring the solutions into shallow dishes in the open air and assisting the removal of the radon by a jet of air which also agitated the solution. A further period of 2 hr. was allowed for the decay of most of the active deposits remaining in solution, and then the estimation of H_2O_2 was carried out colorimetrically as its titanium compound with a Hilger absorptiometer.

The concentration of the enzyme solution was 0·1% (w/v). In order to render the H_2O_2 formed by α-radiation less liable to decomposition, the enzyme solution and the control were adjusted to pH 3·8 by addition of H_2SO_4. This pH is on the acid side of the pH of minimum solubility and of the isoelectric point of the enzyme. All solutions contained O_2 in equilibrium with air.

Control experiments were made to determine the loss of water by evaporation during the blowing off of the radon.

RESULTS

The results of the experiments are set out in Table 1. As in the earlier experiments, the radiation received by the solution can be divided into three parts: (1) Radiation from active deposits laid down on the splinters of the glass bulb between the time of pumping the radon and breaking the bulb (half of this radiation is assumed to be absorbed by the splinters). (2) Radiation from radon and its active deposit during the time the radon is in solution. (3) Radiation from the active deposits remaining in the solution after removal of the radon, and continuing up to the moment of making the optical density measurement.

Table 1. *Effect of carboxypeptidase on the hydrogen peroxide yield from water irradiated at* pH 3·8 *by α-rays*

(Significance test of difference of ionic yield gives $d/\sigma = 1{\cdot}02$ and $P = 0{\cdot}30$.)

	Carboxypeptidase	Water
Time of breaking bulb (t_1) (min.)	2·7	3·2
Time of removing radon (t_2) (min.)	107	114
Time of measurement (t_3) (min.)	250	262
Initial amount of radon (mc.)	61·2	60·0
Volume of irradiated solution (ml.)	3·07	2·85
Volume of solution, after evaporation, used in measurement (ml.)	2·34	0·66
Estimated loss of water by evaporation (ml.)	0·20	0·05
Amount of H_2O_2 measured (μg.)	103·5	29·5
Fraction of H_2O_2 measured	0·83	0·25
Amount of H_2O_2 in irradiated solution (μg.)	125·0	118·1
α-Energy liberated in solution (joules)	43·0	44·9
β-Ray correction:		
β-Energy liberated in solution (joules)	2·2	2·3
Estimated H_2O_2 due to β-radiation (μg.)	3·1	3·2
Amount of H_2O_2 due to α-radiation (μg.)	121·9	114·9
Ionic yield for α-radiation	0·264	0·239
Estimated errors (%) of:		
Absorption measurement	2·4	8·5
Fractionation of solution	2	3
Relative amounts of radon	1	1
Total error (%)	3·3	9·6

The α-particles come from the disintegration of radon and the active deposits of RaA, RaC and RaC′, and in calculating the energy delivered by them, account must be taken of the growth and decay of these products. This can most conveniently be done by calculating an auxilliary function, $F(t)$, which is the total energy delivered to a solution by the α-particles from radon and its products up to time t, after starting at time $t = 0$ with radon free from active deposits.

It is convenient to normalize the function so that it has the value unity after full decay of the radon, and to use the fact that 1 mc. of radon going to full decay gives in α energy, from itself and its products, 54·09 joules. The function $F(t)$ has the form

$$F(t) = 1 - \sum_{r=1}^{4} \gamma_r e^{-\lambda_r t}$$

with the following values for the constants λ and γ:

	1 (Rn)	2 (RaA)	3 (RaB)	4 (RaC)
λ	0·000126	0·227	0·0259	0·0352 min.$^{-1}$
γ	+1·003800	−0·000179	−0·008369	+0·004745

It is then easy to show that the total energy delivered per mc. of radon to the experimental solutions, assuming half the radiation from the active deposits on the glass splinters to be absorbed by the glass, is $54{\cdot}09 \times E$ joules, where

$$E = \tfrac{1}{2}[F(t_3) + e^{-\lambda_1 t_1} F(t_3 - t_1) - F(t_1)] - e^{-\lambda_1 t_2} F(t_2 - t_3)$$

and t_1, t_2 and t_3 are the times in minutes after pumping the radon to breaking the bulb, removing the radon and estimating the H_2O_2 produced. In these calculations it is assumed that the decay of short-lived RaC′ (half life about 10^{-6} sec.) follows instantaneously after that of the parent RaC, and that the radiations from the long lived deposits RaD, etc., can be neglected.

The solutions are, in addition to the α-rays, irradiated by the β-particles, and the energy contributed by these is about 5·2% of the α-energy. Assuming that the β-radiation acts independently of the α-radiation, the amount of H_2O_2 produced by the β-radiation has been estimated from the experiments of Bonet-Maury & Lefort (1950), due allowance being made for dose rate and the pH of the solution.

In calculating the ionic yield, the energy expended per ion-pair was taken as 32·5 eV., leading to $1{\cdot}92 \times 10^{17}$ ion-pairs per joule.

The errors of the different parts of the experiment have been estimated and are shown in Table 1. The difference of the ionic yield with and without the enzyme, when compared with the estimated error of the difference was not significant.

DISCUSSION

The results show that the addition of the enzyme carboxypeptidase does not reduce the yield of H_2O_2 formed by irradiation of water with α-particles. Thus the second hypothesis put forward in the introduction as an explanation of the low α efficiency for inactivation of the enzyme must be discarded and the result is consistent with the first hypothesis.

It will be seen from Table 1 that an allowance of approximately 3% has been made for the H_2O_2 produced by the β-radiation which accompanies the α-radiation. It is difficult to evaluate this correction with accuracy because of the complexity of various competing reactions. The α-radiation alone gives rise to a final H_2O_2 concentration of around 1000 μmole/l. which, according to Bonet-Maury & Lefort (1950), approaches the steady state concentration of H_2O_2 resulting from the exposure of aerated water to X-radiation and which presumably is also comparable with the steady state concentration resulting from exposure to β-radiation.

There is, therefore, some doubt as to whether the β-radiation has augmented or decreased the H_2O_2 concentration produced by the α-radiation in pure water. It is also clear that when the concentration of H_2O_2 is such that there is an approximate balance between its rate of production and rate of destruction by radicals formed by β-radiation, the introduction of a solute, such as carboxypeptidase, may disturb the balance in either direction on account of differences of affinity for the different types of radicals. The slightly greater yield of hydrogen peroxide observed by irradiating a carboxypeptidase solution as compared with that obtained from irradiated pure water, could, therefore, have arisen secondarily through the disturbing effect of the small amount of β-ray energy accompanying the α-radiation. As mentioned earlier in the text, however, the difference is not statistically significant.

In conclusion we can say that the experiments reported here confirm the view suggested in the earlier publication (Dale *et al.* 1949) that the production of H_2O_2 by α-radiation takes place in the core of the track and that the small amount of inactivation of carboxypeptidase by α-radiation is due to the very low energy electrons or δ-rays which branch off as spurs from the α-track.

SUMMARY

The yield of hydrogen peroxide produced by the action of α-rays from radon on water and on a 0·1% solution of the enzyme carboxypeptidase have been compared and no significant difference found. This result confirms the view suggested in an earlier publication that the production of hydrogen peroxide by α-radiation takes place in the core of the track and that the small amount of inactivation of carboxypeptidase by α-radiation is due to the δ-rays which branch off as spurs from the α-track.

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Studies in the Biochemistry of Micro-Organisms

88. PALITANTIN. PART 2. FURTHER DERIVATIVES AND DEGRADATION PRODUCTS

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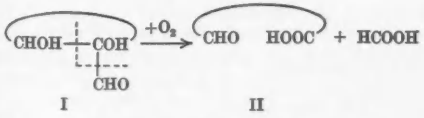
The isolation of the mould metabolic product, palitantin, $C_{14}H_{22}O_4$, obtained from *Penicillium palitans* Westling, was described by Birkinshaw & Raistrick (1936). The product was characterized as an aldehyde containing two hydroxyl groups and apparently possessing two double bonds. The function of the fourth oxygen atom was not determined. Further derivatives and degradation products of palitantin have been prepared and are described in the present communication.

In the earlier paper (Birkinshaw & Raistrick, 1936) palitantin was described as optically inactive, since under the experimental conditions employed the rotation observed, $+0{\cdot}01°$, was regarded as zero within the limits of experimental error. Some of the derivatives and breakdown products have now been found to show considerable optical activity. When palitantin was re-examined under more favourable conditions it was found to have a rotation of $[\alpha]_{5461}^{23°} + 4{\cdot}4°$ (*c*, 0·8 in $CHCl_3$).

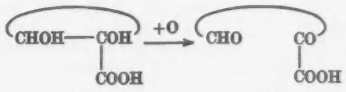
In a quantitative study of the periodate oxidation of palitantin, tetrahydropalitantin, and tetrahydropalitantic acid, the following points have been established. (1) Palitantin and tetrahydropalitantin react with potassium periodate using the equivalent of 2 atoms of oxygen. (2) Two acidic groups are produced, one of which readily lactonizes. (3) An aldehyde group is also produced. (4) The aldehyde and lactonic groupings are constituents of a C_{13} molecule, the other carbon atom being detached as formic acid. (5) With tetrahydropalitantic acid, only one oxygen atom is used, no acidic group is produced but two carbonyl groups are formed.

These facts appear capable of a simple explanation, namely that the reactive grouping in palitantin is part of a ring, and has the form I.



I II

This would be split at the dotted lines by 2 molecules of periodic acid to yield form II plus 1 molecule of formic acid. With tetrahydropalitantic acid, in which the aldehyde group of tetrahydropalitantin has been oxidized to carboxyl, the starting material would be represented by form III which on oxidation with 1 molecule of periodic acid would pass to IV.



III IV

Since the C_{13} acid lactone from tetrahydropalitantin gives rise on nitric acid oxidation to a *n*-heptyl succinic acid, tetrahydropalitantin would appear to consist of a 6-carbon ring with two side chains, one being a CHO group and the other a *n*-heptyl chain. One double bond in palitantin must be situated $\gamma\delta$ to the terminal methyl group of the C_7 chain to account for the production of *n*-butyraldehyde on ozonolysis. The position of the second double bond, if present, is still obscure, likewise the function of the fourth oxygen atom which is presumably attached to a ring carbon atom.

The examination of palitantin continues and it is hoped to discuss its constitution more fully in a later paper.

The dextrorotatory acid which was obtained, together with palitantin, as a metabolic product of the type strain of *Penicillium palitans* (Birkinshaw & Raistrick, 1936, p. 803), has been further examined. It crystallizes from water in colourless needles, m.p. 134·5°, $[\alpha]_{5461}^{24°} + 82°$ (in $CHCl_3$). It has the empirical formula $C_{14}H_{20}O_4$, titrates as a monobasic acid and gives a reddish-purple colour with ferric chloride. Curtis, Hemming & Smith (1951) have recently isolated an antifungal metabolic product, frequentin, from a strain of *Penicillium frequentans* Westling, which was also found to produce palitantin. From the published description frequentin appeared to be very similar to the acid product from *P. palitans*. By a comparison of samples it has been established that the two products are in fact identical; there was no depression of melting point on mixing. The acidic product from *P. palitans* is

therefore frequentin. Curtis & Duncanson (1952) have now shown that frequentin and palitantin are closely related in structure.

EXPERIMENTAL

All melting points are uncorrected.

Properties of palitantin and its derivatives

Specific rotations. Birkinshaw & Raistrick (1936) stated that palitantin is optically inactive. This opinion was based on the fact that a solution of palitantin (0·1505 g.) in $CHCl_3$ (15 ml.) had an observed average rotation of +0·01° (limits of five readings 0·00° to +0·02°) in a 10 cm. tube in the Hg green light (5461 A.) at 20°. This corresponds to $[\alpha]^{20°}_{5461} = +1·0°$, which was considered to be within the limits of experimental error.

When it was subsequently observed that derivatives of palitantin have relatively high specific rotations, the specific rotation of palitantin was redetermined. A solution of palitantin in $CHCl_3$ was exhaustively extracted with aqueous alkali to remove traces of frequentin (see p. 276). The purified $CHCl_3$ solution was evaporated to dryness *in vacuo*, and the residual palitantin was crystallized a number of times from boiling water. A solution of the purified palitantin (0·3941 g.) in $CHCl_3$ (50 ml.) in a 40 cm. tube had an observed average rotation of +0·14° (limits of seven readings +0·13° to +0·15°), corresponding to $[\alpha]^{23°}_{5461} = +4·4°$.

The observed specific rotations of palitantin and some of its derivatives are recorded in Table 1.

Table 1. *Specific rotation of palitantin and its derivatives*

Substance	Temperature (°)	Solvent	Vol. of solvent (ml.)	Wt. of substance (g.)	Length of tube (cm.)	$[\alpha]_{5461}$ (°)
Palitantin	23	$CHCl_3$	50	0·3941	40	+ 4·4
Palitantin monophenylhydrazone	24	Pyridine	1	0·0088	5 microtube	−93
Palitantin bis-*p*-bromobenzoate	24	Ethanol	1	0·0110	5 microtube	−13
Palitantic acid	20	Ethanol	1	0·0100	5 microtube	−74
α-Hexahydropalitantin	24	Ethanol	1	0·0074	5 microtube	−54
β-Hexahydropalitantin	24	Ethanol	1	0·0108	5 microtube	−72
Tetrahydropalitantic acid	24	Ethanol	1	0·0102	5 microtube	−90
Tetrahydropalitantin bis-*p*-bromobenzoate	23	Ethanol	25	0·0666	20	− 2
Tetrahydropalitantin	24	Ethanol	50	0·5836	40	−43·1

Carbon-methyl groups

Palitantin. 1·19 and 1·18 mol. of acetic acid were produced per mol. of palitantin in the Kuhn-Roth micromethod. (Estimations by Dr Roth.)

A mixture of palitantin (0·2589 g.), CrO_3 (12 g.), $K_2Cr_2O_7$ (3 g.), water (30 ml.) and H_3PO_4 (sp.gr. 1·75, 20 ml.) was heated on a boiling-water bath under reflux for 3·5 hr. (Karrer, Helfenstein, Wehrli & Wettstein, 1930). A distinct smell of butyric acid was observed. The oxidation mixture, contained in a Claisen flask, was distilled *in vacuo* until the whole of the volatile acids had been removed in the distillate. The total volatile acid (10·23 ml. of 0·1 N) corresponds to 1·003 mol. of acetic acid/mol. of palitantin.

Tetrahydropalitantin. Tetrahydropalitantin (0·2876 g.) on similar oxidation yielded 11·03 ml. 0·1 N volatile acid, equivalent to 0·989 mol. of acetic acid per mol. of tetrahydropalitantin.

Reaction of palitantic acid with sodium amalgam and of palitantin with maleic anhydride

With sodium amalgam. Palitantic acid (1 g.) was treated with Na amalgam (2·5 % Na; 100 g.) and water with vigorous stirring. The reaction was maintained about neutral by frequent additions of H_2SO_4. The solution was filtered whilst slightly alkaline and acidified. Crystals (0·8 g.) were obtained, which proved to be unchanged palitantic acid. The reduction was therefore continued using a further 400 g. of amalgam over a period of 2 days. Unchanged palitantic acid (0·65 g.) was again recovered, an almost quantitative recovery when allowance is made for the solubility of palitantic acid. A further attempt to obtain reduction in acidic ethanol also failed. This renders unlikely the presence of a conjugated double bond or of a grouping capable of keto-enol tautomerism.

Action of maleic anhydride on palitantin. A solution of palitantin (0·2 g.) and maleic anhydride (0·1 g.) in benzene (100 ml.) was refluxed for 1·5 hr. On cooling, crystals separated and were collected, washed with benzene and dried, wt. 0·13 g., m.p. 164–165° not depressed on admixture with palitantin. Hence palitantin does not appear to contain a conjugated double bond system.

Derivatives of palitantin, tetrahydropalitantin and palitantic acid

Monoiodopalitantin. 0·1 N Aqueous I_2 solution (100 ml.) was added to a solution of palitantin (0·5 g.) in ethanol (25 ml.). The solution, from which crystals soon began to separate, was held for 1 hr. initially at room temperature and finally in the refrigerator. The excess I_2 was removed by titration with chilled 0·1 N-$Na_2S_2O_3$. I_2, equivalent to 40 ml. 0·1 N, was absorbed, corresponding almost quantitatively to

the uptake of 2 atoms of I/mol. of palitantin. The slightly yellow crystals (0·47 g.) were collected, dried and recrystallized from aqueous ethanol (2 vol. water: 1 vol. ethanol). Beautiful long colourless silky needles (0·30 g.) of *monoiodopalitantin* separated, m.p. 136° (sharp, but decomposing to a black liquid which froths up the melting point tube). (Found: C, 44·5, 44·6; H, 5·7, 5·7; I, 33·7, 33·4. $C_{14}H_{21}O_4I$ requires: C, 44·2; H, 5·6; I, 33·4%.) Monoiodopalitantin is thus derived according to the equation

$$C_{14}H_{22}O_4 + I_2 \rightarrow C_{14}H_{21}O_4I + HI.$$

Monoiodopalitantic acid. 0·1 N Aqueous I_2 solution (20·0 ml.) was added to a solution of palitantic acid (0·0540 g.) in 0·1 N aqueous NaOH (2·05 ml.), and the whole made up to a total volume of 50·0 ml. After 1 hr. the residual I_2 was estimated by titration with 0·1 N-$Na_2S_2O_3$. Iodine equivalent to 1·95 atoms/mol. of palitantic acid was absorbed. The titrated solution was acidified with 0·1 N-HCl (2 ml.). After chilling, colourless needles (0·023 g.) of *monoiodopalitantic acid* separated and were collected, washed and dried. Melting point 135·5°, darkening on melting, but not blackening or frothing as did monoiodopalitantin. (Found: C, 42·1, 42·3; H, 5·5, 5·6; I, 31·8, 31·5. $C_{14}H_{21}O_5I$ requires: C, 42·4; H, 5·3; I, 32·0%.)

Palitantin monoxide. When palitantin is treated with a $CHCl_3$ solution of camphoric acid peracid at room temperature (3·8 g. in 100 ml. $CHCl_3$ solution as determined by thiosulphate titration—Milas & Cliff, 1933), oxidation takes place corresponding roughly with the oxidation of one double bond in 1 hr. and two double bonds in 20 hr.

Palitantin (0·25 g.) was treated with an excess (25 ml.) of camphoric acid peracid solution in $CHCl_3$ and held for 1 hr. at room temperature. The palitantin gradually dissolved and had completely dissolved in less than 1 hr. The mixture was then thoroughly extracted with aqueous $NaHCO_3$. On removal of the solvent from the $CHCl_3$ layer, a colourless solid remained which was recrystallized from ether (50 ml.). *Palitantin monoxide* was thus obtained as beautiful colourless clusters of needles (0·03 g.), m.p. 144°. It is fairly readily soluble in water. (Found: C, 62·7, 62·9; H, 8·3, 8·3. $C_{14}H_{22}O_5$ requires: C, 62·2; H, 8·2%.)

A solution of palitantin (1 g.) in 100 ml. of a $CHCl_3$ solution of camphoric acid peracid was held at room temperature for 18 hr. The oxygen absorbed corresponded almost exactly to 2 atoms/mol. of palitantin. The oxidation product, isolated as described in the previous paragraph, proved to be mainly an uncrystallizable oil containing 0·06 g. of palitantin monoxide, m.p. 137°. (Found: C, 61·9, 61·8; H, 8·2, 8·0%. $C_{14}H_{22}O_5$ requires: C, 62·2; H, 8·2%.)

Tetrahydropalitantin bis-p-bromobenzoate. A mixture of tetrahydropalitantin (0·25 g.), *p*-bromobenzoyl chloride (0·8 g.) and pyridine (5 ml.) was heated on a boiling-water bath for 10 min. Crystals formed on cooling. Water was added and decanted after a time. The residue was thoroughly extracted with aqueous $NaHCO_3$ and finally with water. The sticky residue was boiled with ethanol (30 ml.) and the residual crystals were separated and identified as bromobenzoic anhydride. By the cautious addition of water to the ethanolic mother liquors a sticky first crop of crystals was obtained which was rejected. The second crop was crystalline and, after recrystallization from water, *tetrahydropalitantin bis-p-bromobenzoate* was obtained as colourless clusters of flattened needles, m.p. 94·5°. (Found: C, 53·9, 53·9; H, 5·2, 5·3; Br, 25·4. $C_{28}H_{32}O_6Br_2$ requires: C, 53·8; H, 5·2; Br, 25·6%.)

Breakdown products and their derivatives

Hydrolysis of the dihydrazide $C_{13}H_{28}O_3N_4$ *obtained by oxidation of tetrahydropalitantin with silver oxide*

A solution of the dihydrazide (Birkinshaw & Raistrick, 1936, p. 807). (0·025 g., m.p. 190°) in 10 N-H_2SO_4 (5 ml.) was boiled under reflux for 0·5 hr. After cooling, the hydrolysate was diluted with water (20 ml.) and thoroughly extracted with ether. On removal of the solvent and thorough drying *in vacuo*, a syrup (0·0185 g.) remained and showed no signs of crystallization. It was dissolved in ethanol (1 ml.) and titrated in the cold to phenolphthalein with 0·1 N-NaOH; 0·81 ml. of 0·1 N-NaOH were required, corresponding to an equivalent of 228. A mono-acid monolactone of the formula $C_{13}H_{22}O_4$, titrating as a monobasic acid, requires 242.

An excess (2·00 ml.) of 0·1 N-NaOH was now added and the whole was heated on the water bath for 1 hr. After cooling, 1·27 ml. of 0·1 N-HCl was required to neutralize to phenolphthalein. Hence the additional alkali used in the hydrolysis = 0·73 ml., giving a total amount of alkali used of 0·81 + 0·73 ml. = 1·54 ml. 0·1 N. This corresponds to a total equivalent of 120. $C_{13}H_{22}O_4$, i.e. the mono-lactone of $C_{13}H_{24}O_5$, titrating as a dibasic acid requires an equivalent of 121.

Oxidative fission of palitantic acid. Formation of n-*butyraldehyde*

With $KMnO_4$. A solution of $KMnO_4$ (2 g.) in water (200 ml.) was added dropwise to a solution of palitantic acid (1 g.) in water (150 ml.) and N-NaOH (4 ml.). During the oxidation a brisk current of air was passed through the solution, and the issuing gas was scrubbed through bubblers containing Brady's reagent (2:4-dinitrophenylhydrazine in 2 N-HCl). The resulting yellow precipitate was collected, dried and crystallized from methanol. Yellow needles, m.p. 123·5°, not depressed on admixture with authentic *n*-butyraldehyde 2:4-dinitrophenylhydrazone, m.p. 123°. (Found: C, 47·4, 47·4; H, 4·9, 4·8; N, 21·8, 22·0. Calc. for $C_{10}H_{12}O_4N_4$: C, 47·6; H, 4·8; N, 22·2%.) No other pure products could be isolated from the oxidation solution.

Ozonolysis. Ozonized O_2 was passed for about 7 hr. through a solution of palitantic acid (0·54 g.) in 0·1 N-NaOH (20 ml.) and water (40 ml.). The mixture was now heated to about 60° and a current of air was bubbled through it and then through Brady's reagent. The resulting yellow precipitate proved to be *n*-butyraldehyde 2:4-dinitrophenylhydrazone. 0·42 g. of impure palitantic acid was recovered from the ozonized solution.

Oxidation of the lactonic acid $C_{13}H_{22}O_4$ *from tetrahydropalitantin with nitric acid. Formation of* (+)-n-*heptylsuccinic acid*

The syrupy lactonic acid from tetrahydropalitantin (1·1 g.; Birkinshaw & Raistrick, 1936, p. 807) was heated on the water bath with a mixture of conc. HNO_3 (13 ml.) and water (9 ml.). The reaction was vigorous at first, but gradually slackened and became negligible after 3 hr. The mixture was evaporated on the water bath in an open dish to a syrup. A smell of fatty acids, butyric, hexanoic, etc., was observed. On cooling, a mass of crystals mixed with oil was obtained, wt. 0·90 g. This mass was treated with a little water, well stirred and filtered. The filtrate, which consisted

of an aqueous solution with drops of oil suspended in it, contained much oxalic acid.

The solid (wt. 0·44 g.; m.p. 39–42°) was shown by titration to contain a little unchanged lactonic acid. This was removed by repeated solution of the crystals in the titratable amount of N-NaOH followed by acidification with HCl. The final product, having a titration equivalent of 111, was recrystallized from ether (1 vol.) followed by the addition of light petroleum (b.p. 40–60°; 10 vol.). The oxidation product was thus obtained as colourless slender rods, m.p. 82–84°, not depressed on admixture with synthetic (±)-*n*-heptylsuccinic acid, m.p. 86° (see next section). (Found: C, 61·1, 61·1; H, 9·0, 9·3; equivalent by titration 108·7. Calc. for $C_{11}H_{20}O_4$: C, 61·1; H, 9·3%; equivalent, titrating as a dicarboxylic acid, 108·1.) $[\alpha]_{5461}^{20°} = +33°$ (*c*, 0·97 in ethanol).

Optically active (+)-n-*heptylsuccinic acid dihydrazide.* The oxidation acid (0·2 g.) was esterified with diazomethane and treated with a solution of hydrazine hydrate (50%; 0·5 ml.) in ethanol (1 ml.). The crystals which appeared overnight were recrystallized from ethanol-ether and were obtained as colourless needles, m.p. 166°. (Found by I.C.I. Butterwick Research Laboratories: C, 53·8, 54·0; H, 9·9, 9·8; N, 22·3, 22·5. $C_{11}H_{24}O_2N_4$ requires C, 54·1; H, 9·9; N, 22·9%.)

Synthesis of (±)-n-*heptylsuccinic acid*

Ethyl 2-bromononanoate (α-bromopelargonate) was condensed with ethyl sodiomalonate, and the resulting triester was hydrolysed and heated to eliminate one carboxyl group.

Preparation of ethyl 2-bromononanoate (Auwers & Bernhardi, 1891). Bromine (10 ml.) was added drop by drop to a mixture of nonanoic acid (15·8 g.) and red P (1·04 g.). The mixture was gradually warmed to 90–100° and more Br (2 ml.) was added. When the evolution of HBr had practically ceased (4–5 hr.) the mixture was cooled and ethanol (30 ml.) was added with cooling. Next day the ethyl α-bromopelargonate was extracted with ether and the ethereal solution was washed with water and aqueous Na_2CO_3, dried over Na_2SO_4 and distilled *in vacuo*. The ester (22·45 g.) was collected at 135–142°/15 mm.

Condensation of ethyl 2-bromononanoate with malonic ester and hydrolysis of the resulting triester. Na (1·96 g.) was dissolved in ethanol (27 ml.) and malonic ester (13·6 g. = 11·8 ml.) was added. The mixture was warmed slightly and ethyl 2-bromononanoate (22·4 g.) was added, and the whole was refluxed for 2 hr. The resulting ester was extracted in the usual manner and distilled *in vacuo*. Yield 21·7 g.; b.p. 153°/1 mm.

The ester (2 g.) was heated with KOH (5 g.) and water (3 ml.) in a nickel crucible with constant stirring, the temperature of the metal bath being raised to 300° as rapidly as frothing would permit. The melt was cooled, dissolved in water and acidified with HCl. Colourless plates (1·27 g.) separated, m.p. 126–128°.

Decarboxylation of the tricarboxylic acid. The above acid, m.p. 126–128°, was heated in an oil bath when decarboxylation occurred at the melting point (bath temp. 135–140°). After about 10 min. effervescence had practically ceased. The product which crystallized on cooling was neutralized with aqueous N-NaOH, filtered and acidified with HCl. The resulting emulsion soon crystallized. The crystals were collected, dried and recrystallized from ether-light petroleum.

(±)-n-*Heptylsuccinic acid* was thus obtained as colourless irregular prisms, m.p. 86°; equivalent by titration 110. (Found by I.C.I. Butterwick Research Laboratories: C, 61·0, 61·0; H, 9·0, 9·2. $C_{11}H_{20}O_4$ requires C, 61·1; H, 9·3%; equivalent 113.)

(±)-n-*Heptylsuccinic acid dihydrazide.* This dihydrazide was prepared in the same way as was the dihydrazide of the oxidation product (+)-*n*-heptylsuccinic acid (see above). It formed colourless needles, m.p. 156°. Although there was a difference of 10° in melting point between the synthetic (±) and the natural dihydrazides, there was no depression in melting point on mixing them.

Reactions with periodate

Action of periodate on tetrahydropalitantin

Oxygen utilization. Tetrahydropalitantin (0·0403 g.) dissolved in water (100 ml.) by warming and subsequent cooling was treated with KIO_4 (50 ml. of 0·0125 M) and left overnight. Excess H_2SO_4 and KI were then added and the I_2 liberated was titrated with 0·05 N-$Na_2S_2O_3$. The I_2 deficit as compared with a blank experiment was 6·24 ml. of 0·1 N, equivalent to a disappearance of four atoms I or utilization of two atoms O/mol. of tetrahydropalitantin.

Acidic groups formed. The experiment was repeated with 0·1616 g. of tetrahydropalitantin using the theoretical amount (100 ml.) of KIO_4 (0·0125 M), in order to determine the acid and carbonyl groups present after the reaction. After standing overnight, the solution required 11·54 ml. of 0·1 N-NaOH for neutralization to phenolphthalein. The theoretical figure for 2 equivalents of acid produced in the reaction would be 12·50 ml. The actual figure obtained was always somewhat lower, doubtless owing to lactone formation.

Aldehyde titration

By Doeuvre's method (Doeuvre, 1927). To the neutralized solution was added mercury potassium iodide (3 g.) in a little water with just sufficient KI to obtain complete solution, followed by NaOH (40 ml. of 0·1 N). A yellow precipitate was formed in the solution. After 3 hr., titration with 0·1 N-acetic acid containing equimolar sodium acetate showed the disappearance of 16·05 ml. of alkali. The aldehyde present is equivalent to 5·35 ml. (= ⅓ of 16·05), whereas the theoretical value for 1 CHO = 6·25 ml.

By bisulphite titration (Malaprade (1934) method). The periodate oxidation was repeated (0·1616 g. of tetrahydropalitantin; 100 ml. of 0·0125 M-KIO_4). 11·20 ml. of 0·1 N-NaOH were required for neutralization of the acid produced in the reaction. Bisulphite (30 ml. about 0·2 M) was then added, the solution was left for 1 hr. with exclusion of air, and was then titrated. It required 15·90 ml. of 0·05 M-iodate. The bisulphite taken was equivalent (by titration) to 45·04 ml. of 0·05 M-iodate, the difference being 29·14 ml. Of this 25·00 ml. would be derived from the periodate. The iodate corresponding with aldehyde is, therefore, 4·14 ml. whereas 1 CHO group would require 4·17 ml.

By hydroxylamine titration (Schultes, 1934). The same method of oxidation was followed and the same quantities used, but after removal of the iodate with H_2SO_4 and KI and titration of the liberated I_2 with thiosulphate, the solution was neutralized to bromophenol blue, and a solution of about 1 g. of NH_2OH, HCl neutralized to the same end point was added. The mixture immediately became acid and was

titrated from time to time with 0·1 N-NaOH until permanent neutrality (to the same end point) was reached. The alkali used was 6·7 ml., whereas 1 CHO group requires 6·25 ml.

There is ample evidence to show the production (or persistence) of 1 CHO group on periodate oxidation.

Production of lactone group. The production of a lactone group is shown more clearly in the following experiment. Tetrahydropalitantin (0·25 g.) in 25 ml. of methanol was treated with KIO_4 (0·5 g.) in water (125 ml.). A turbidity appeared in 5–10 min. which gradually increased. After standing overnight the mixture was titrated to phenolphthalein. It used 16·8 ml. of 0·1 N-NaOH to the first pink flush, but required 5–6 hr. to reach a permanent end point, when 20·5 ml. of NaOH had been utilized, the theoretical figure for 2 equiv. of acid produced being 19·4 ml. After standing overnight a further 0·30 ml. of 0·1 N alkali was required.

Characterization of the periodate oxidation product of tetrahydropalitantin. The product obtained by the oxidation of tetrahydropalitantin (0·25 g.) with KIO_4 (0·5 g.) was extracted with ether, suspended in water (100 ml.) and treated with Doeuvre's reagent (0·45 g. of HgI_2, 2 g. of KI in a little water). Then N-NaOH (10 ml.) was introduced, the mixture was well shaken and left overnight. The precipitated Hg was filtered off and the solution titrated. It required 6·40 ml. of N-HCl; the acid produced in the reaction is therefore 3·60 ml. (1 CHO group requires 3·88 ml.). The solution was now acidified with HCl and extracted with ether. The ether extract was esterified by means of diazomethane. The resulting oil (which contained a little Hg salt) was treated with ethanol (2 ml.) and hydrazine hydrate (50%, 1 ml.). The solid mass of crystals obtained (0·17 g.) was drained at the pump, and recrystallized from ethanol giving 0·12 g. of pure product of m.p. 190°. This gave no depression when mixed with the product $C_{13}H_{28}O_3N_4$ obtained previously by the oxidation of tetrahydropalitantin with Ag_2O (Birkinshaw & Raistrick, 1936, p. 807).

In a larger scale experiment tetrahydropalitantin (1·613 g.) was oxidized by KIO_4 (2·875 g.) and the aldehydic product further oxidized with Doeuvre's reagent as above. In this case the mercury precipitated was weighed and acid produced in the Doeuvre reaction was determined (Hg 1·23 g. 1 CHO requires 1·25 g.; acid formed 17·9 ml. 1 CHO requires 18·75 ml.).

The solution was acidified to Congo red with H_2SO_4 and extracted with $CHCl_3$ (4 × 2 l.). After removal of solvent *in vacuo* the residue (1·22 g.) was taken up in ether, filtered from a small amount of HgI_2, taken up by the $CHCl_3$ and esterified by means of diazomethane. The product, distilled *in vacuo* afforded 0·84 g. of distillate b.p. 175°/3 mm. with methoxyl content 12·6%. $C_{14}H_{24}O_4$ requires (for 1 OMe) 12·1%. This represents the monomethyl ester of the lactone acid.

The distillate (0·79 g.) was treated with phenylhydrazine (0·5 g.) in ethanol (2 ml.) and heated under reflux for 2 hr. The product was treated with ether and the solvent allowed to evaporate slowly. A small amount of crystals was obtained, m.p. 192°, raised on recrystallization to 206°. (Found: C, 67·8; H, 8·2; N, 12·8. $C_{25}H_{36}O_3N_4$ requires C, 68·1; H, 8·2; N, 12·7%. The product is evidently the *bisphenylhydrazide* of the lactonic acid $C_{13}H_{22}O_4$.

Action of periodate on palitantin

Palitantin (0·0594 g.) in ethanol (25 ml.) was treated with KIO_4 (0·0125 M; 100 ml.) and H_2SO_4 (2 N; 10 ml.). After 20 hr. at room temperature excess KI was added and the I_2 was titrated with 0·1 N-$Na_2S_2O_3$. The I_2 deficit as compared with a blank was 13·03 ml. 0·1 N corresponding with 5·74 atoms I/mol. of palitantin. Part of the I_2 uptake is due to I_2 substitution, which is a characteristic property of palitantin and presumably of the fission product.

When the theoretical amount of KIO_4 for an uptake of 4 I atoms (=2O) was used and there was no subsequent liberation of free I_2 the results were similar to those obtained with tetrahydropalitantin.

Evidence of lactone formation. Palitantin (1 g.) in methanol (100 ml.) treated with KIO_4 (2 g.) in water (600 ml.) remained clear. After 24 hr. it required 6·4 ml. of N-NaOH to the first pink flush of phenolphthalein gradually increasing to 7·95 ml. over 3 hr. The theoretical value for 2 equivalents of acid produced is 7·87 ml. This is again evidence for one product being a lactone.

Aldehyde production. The reaction mixture was acidified with 10 ml. of 2 N-H_2SO_4 and extracted with ether. The oily residue obtained on evaporation of the ether was suspended in water (about 200 ml.) and neutralized to bromophenol blue. After addition of a solution of $NH_2OH.HCl$ (2 g.) at the same pH the solution was titrated at intervals until a permanent end point was reached, matching to the same arbitrary standard. The NaOH required was 3·4 ml., the theoretical amount for 1 aldehyde group being 3·88 ml. Practically all the emulsion dissolved leaving only a trace of turbidity.

Oxidation of aldehyde group and characterization of product. The product from the KIO_4 (2 g.) oxidation of palitantin (1 g.) was extracted with ether after acidifying. The residual syrup after removal of ether was oxidized with Doeuvre's reagent. Barium acetate was added and the oxidation product was precipitated as the barium salt by addition of 4 vol. of ethanol. The Ba precipitate was collected, suspended in water, acidified with HCl and the acid product extracted with ether. The product was then esterified by means of diazomethane and treated with ethanol (4 ml.) and hydrazine hydrate (50%; 2 ml.). The crystals obtained (0·47 g.; m.p. 202–204°) showed no depression when mixed with the dihydrazide $C_{13}H_{24}O_2N_4$ obtained by oxidation of palitantin with Ag_2O as previously described (Birkinshaw & Raistrick, 1936).

Action of periodate on tetrahydropalitantic acid

Utilization of oxygen. Tetrahydropalitantic acid (0·0857 g.) suspended in water (25 ml.) was neutralized to phenolphthalein to bring it into solution and treated with KIO_4 (0·0125 M; 50 ml.) and left overnight. Excess of H_2SO_4 and KI were then added and the liberated I_2 was titrated with $Na_2S_2O_3$. The deficiency of I_2 was 6·44 ml. of 0·1 N as compared with a blank, corresponding with a utilization of 2·06 atoms of I ($\equiv$1·03 atoms of O).

Determination of acid and carbonyl groups. Tetrahydropalitantic acid (0·1713 g.) was dissolved in the theoretical amount of NaOH (0·1 N; 6·25 ml.) and KIO_4 (0·0125 M; 50 ml.; i.e. the theoretical amount) was added. After 2 days it required 0·11 ml. of 0·1 N-NaOH to neutralize to phenolphthalein, i.e. no acid is formed in the reaction. To the titrated solution were added H_2SO_4 (0·1 N; 50 ml.) and

excess KI. Titration with $Na_2S_2O_3$ showed a deficiency of 12·48 ml. of 0·1 N-I_2 corresponding with 2 atoms of I (theoretical 12·50 ml.). The solution was then treated with bromophenol blue and brought to an arbitrary end point (faint purple colour) with NaOH. NH_2OH, HCl (1 g. in 10 ml. of water) neutralized to the same end point was added. After 2 hr. the alkali required to establish the same end point was 11·5 ml. of 0·1 N-NaOH. The theoretical value for two aldehyde groups is 12·5 ml. Thus two carbonyl groups are produced in the reaction, provided no such groups titratable with NH_2OH are present in the original tetrahydropalitantic acid. The absence of such groups was shown by the addition of the NH_2OH reagent to another sample of the acid. No acidity was produced.

Isolation of frequentin

The crude crystalline product (11·75 g.) obtained by $CHCl_3$ extraction of the culture solution from two batches of *P. palitans* type strain (Birkinshaw & Raistrick, 1936) was dissolved in $CHCl_3$ (500 ml.) and 100 ml. of water were added. N-NaOH was added dropwise with vigorous shaking until the aqueous layer was just alkaline to phenolphthalein. This required 11·5 ml. of N-NaOH. The aqueous layer was separated and acidified with N-HCl (12 ml.). The oil precipitated solidified on chilling and was crude frequentin, wt. 2·52 g. A small amount was recovered from the mother liquor.

Frequentin was difficult to recrystallize as it decomposed on heating for any length of time. It was purified for analysis by recrystallizing in small amounts from boiling water, with addition of decolorizing charcoal. Frequentin crystallized in colourless needles, m.p. 134·5° (decomp.), $[\alpha]_{5461}^{20°}+82°$ in $CHCl_3$. On microtitration 10·260 mg. required 2·80 ml. N/70-NaOH, equiv. 257. (Found: C, 66·0, 66·0, 66·5, 66·4; H, 7·7, 7·8, 8·2, 8·2. Calc. for $C_{14}H_{20}O_4$: C, 66·6; H, 8·0%, equiv. 252.) Frequentin gives a reddish purple colour with $FeCl_3$ in aqueous or alcoholic solution and yields a precipitate with Brady's reagent.

A comparison of our product obtained from *P. palitans* Westling with frequentin obtained from *P. frequentans* (Curtis *et al.* 1951), kindly carried out by Mr P. J. Curtis, indicated that the two products were identical since there was no depression in melting point on mixing.

SUMMARY

1. A number of derivatives and breakdown products of palitantin have been prepared and are described.

2. Palitantin, contrary to the previous statement (Birkinshaw & Raistrick, 1936), has a slight but definite optical activity.

3. The oxidation of palitantin and its derivatives with periodate has been studied in a quantitative manner.

4. Tetrahydropalitantin probably consists of a 6-carbon ring with a *n*-heptyl and a formyl group as substituents and with hydroxyl groups situated α and β to the formyl group. In palitantin one double bond is in the C_7 chain in the γ position to the terminal methyl group. The function of the fourth oxygen atom and position of the second double bond (if present) are still obscure.

5. The acidic substance which accompanies palitantin as a metabolic product of *Penicillium palitans* is identical with frequentin isolated from *Penicillium frequentans* by Curtis *et al.* (1951).

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A Structural Relationship between Frequentin and Palitantin

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Birkinshaw & Raistrick (1936), in describing the isolation of palitantin ($C_{14}H_{22}O_4$) from *Penicillium palitans*, mention the occurrence of a dextrorotatory acid in concentrates from one strain. Curtis, Hemming & Smith (1951) isolated a substance named frequentin ($C_{14}H_{20}O_4$) together with palitantin from certain atypical strains of *Penicillium frequentans*. Subsequently it was pointed out to us by Dr J. H. Birkinshaw (cf. Birkinshaw, 1952, preceding paper) that the dextro-rotatory acid was very probably identical with frequentin, and we have established this by a mixed melting point determination and by comparing the infrared spectra of the two substances.

In common with palitantin, frequentin has an aldehyde group, an ethylenic double bond or bonds

and three active hydrogens, but is distinguished from it by the presence of an enolic hydroxyl. This paper presents evidence that the carbon skeletons and the positions of the oxygen atoms are identical.

Reduction of frequentin and palitantin

Frequentin was reduced by sodium amalgam in acid solution giving a tetrahydro-derivative ($C_{14}H_{24}O_4$), m.p. 170–171°, which no longer contained a formyl group, or an enolic hydroxyl. Palitantin was also reduced by sodium amalgam giving dihydropalitantin ($C_{14}H_{24}O_4$), m.p. 169°. No depression of melting point occurred on mixing these reduction products.

Infrared spectra of palitantin, frequentin and dihydropalitantin

The spectra of dihydropalitantin and the tetrahydro-derivative of frequentin are the same in all respects. Hence these two substances are identical, and it follows that palitantin and frequentin have the same carbon skeleton and that their oxygen atoms are in corresponding positions.

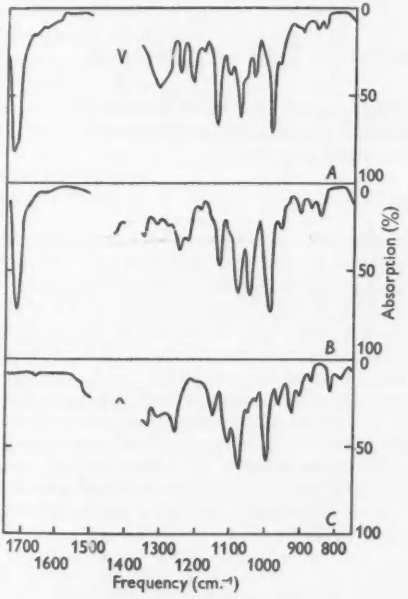

Fig. 1. Infrared absorption spectra. *A*, frequentin; *B*, palitantin; *C*, dihydropalitantin.

The absorption curves, from 750 to 1750 cm.$^{-1}$ of frequentin, palitantin and dihydropalitantin are shown in Fig. 1. Bands due to C=O stretching modes occur in the 6 μ. (1600 cm.$^{-1}$) region; thus as dihydropalitantin has no band in this region, carbonyl groups are absent. Frequentin has an absorption band at 1732 cm.$^{-1}$ with a slight shoulder at about 1720 cm.$^{-1}$, whilst palitantin has a band at 1718 cm.$^{-1}$. This confirms that there are at least two carbonyl groups in frequentin, and at least one in palitantin. The fact that these frequencies are above 1710 cm.$^{-1}$, together with chemical evidence, indicates that unless the carbonyl groups form part of a strained ring, they are not conjugated with other double bonds.

There are quite strong similarities between the spectra of the three compounds, some bands being traceable through them all with only slight modifications of their frequencies. As would be expected, in the region 1000–1200 cm.$^{-1}$ marked differences appear. In this region absorptions due to vibrational modes involving deformation of alcoholic O—H groups occur, and whilst three fairly strong bands are present in all three spectra (i.e. near 1000 cm.$^{-1}$, near 1080 cm.$^{-1}$ and near 1140 cm.$^{-1}$), variations in this frequency range are apparent.

The 3 μ. (3300 cm.$^{-1}$) regions in which C—H and O—H stretching frequencies occur are not shown in the diagram, but bands due to alcoholic hydroxyl groups occur at the following frequencies: frequentin, 3420 cm.$^{-1}$; palitantin, 3370 cm.$^{-1}$; dihydropalitantin, 3350 cm.$^{-1}$.

EXPERIMENTAL

Impure samples of frequentin and palitantin were freed from each other by virtue of the fact that frequentin alone is extracted from $CHCl_3$ solution by Na_2CO_3 solution.

Reduction of frequentin. Frequentin (250 mg.) was dissolved in hot water (500 ml.), and, after cooling, 25 g. KH_2PO_4 were added, then 100 g. of 2·5% Na amalgam in about thirty portions during 2 hr. The mixture was extracted three times with 200 ml. $CHCl_3$, and the extracts after shaking with N-Na_2CO_3 solution saturated with NaCl were combined and concentrated giving colourless plates (35 mg.) m.p. 164–165°. One recrystallization raised the melting point to 170–171°, but no change was observed on further recrystallization. (Found C, 65·3; H, 9·4; mol.wt. 283. $C_{14}H_{24}O_4$ requires C, 65·6; H, 9·4%; mol. wt., 256.) The compound did not reduce ammoniacal Ag_2O nor did it give a colour with $FeCl_3$. It decolorized a solution of Br_2 in glacial acetic acid.

Reduction of palitantin. Palitantin (500 mg.) was dissolved in ethanol (50 ml.) and 2·5% Na amalgam (100 g.) with a slight excess of N-H_2SO_4 was added in ten portions during 6 hr. After standing 1 hr. the mixture was boiled and then treated similarly to that from frequentin. Yield 154 mg., m.p. 162–164°. (Found C, 65·7; H, 9·4. $C_{14}H_{24}O_4$ requires C, 65·6; H, 9·4%.) Recrystallization from water or $CHCl_3$ was unsatisfactory. A sample (m.p. 165°) on admixture with a sample of the tetrahydro derivative of frequentin (m.p. 166–167°), melted at 167°. Purification by fractional extraction between $CHCl_3$ and water gave a solid, m.p. 169°.

Oxidation of dihydropalitantin. The reduction product was found to oxidize slowly in air at room temperature, and after

several weeks a smell like that of butyric acid was noticed. The samples prepared for analysis in the above work were therefore kept *in vacuo* or in N_2.

Infrared spectra

Measurements were made with a modified Hilger D. 209 spectrometer, fitted with a rock-salt prism and working as a single beam instrument. Absorption due to atmospheric water vapour was minimized by enclosing the instrument in an air-tight Perspex box through which dry air was circulated. The samples were ground to fine powders which were moistened with Nujol, the resulting mulls being held as thin films between rock-salt plates for examination.

The intensity of the radiation incident on the samples was determined in the usual way by replacing the samples with two rock-salt plates of the same thickness as those used for supporting the mulls. Absorption curves were then drawn (see Fig. 1), the gaps in which are due to regions of Nujol absorption. For calibration of the spectrometer, hydrocarbon bands of known frequency were used in conjunction with bands due to CO_2 and a small amount of water vapour present in the instrument. Frequencies below 1750 cm.$^{-1}$ are accurate to ± 3 cm.$^{-1}$, and in the 3300 cm.$^{-1}$ region to ± 10 cm.$^{-1}$.

SUMMARY

The mould metabolites frequentin ($C_{14}H_{20}O_4$) and palitantin ($C_{14}H_{22}O_4$) obtained from *Penicillium palitans* and some atypical strains of *Penicillium frequentans* both give dihydropalitantin ($C_{14}H_{24}O_4$) on reduction with sodium amalgam. Hence the carbon skeletons and positions of the oxygen atoms in the two metabolites are identical.

We are indebted to Mr J. F. Grove for valuable advice, to Dr J. H. Birkinshaw for a sample of the dextrorotatory acid, and to the Directors of I.C.I. Ltd. (Plastics Division) and Mr H. A. Willis for spectrographic facilities.

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Oxidation of Pyruvic Acid Oxime by Soil Organisms

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The possibility that hydroxylamine might be an intermediate in the oxidation of ammonia to nitrite by *Nitrosomonas* has often been discussed. There has been, however, no direct evidence to confirm this view and, indeed, hydroxylamine is well known to be toxic in low concentrations to these and many other organisms. Recently, Lees & Quastel (1946), working on the hypothesis that the hydroxylamine formed might be bound as an oxime, reported that the perfusion of an equimolar mixture of sodium pyruvate and hydroxylamine over a soil enriched with nitrifying organisms through previous perfusions of ammonium chloride, resulted in the formation of nitrate. They suggested that the equilibrium of the reaction between sodium pyruvate and hydroxylamine might be such that only a very low concentration of free hydroxylamine was present at any instant. This

$$CH_3.CO.COONa + NH_2OH \rightleftharpoons CH_3.C:(N.OH).COONa + H_2O$$

concentration might be sufficiently low to allow oxidation of the free hydroxylamine and yet eliminate its toxic effects. Subsequently, Quastel & Scholefield (1949) were able to show that neither *Nitrosomonas* nor *Nitrobacter* was responsible for oxidation of pyruvic acid oxime (hereafter called pyruvic-oxime) and predicted the existence of a separate enzymic mechanism. The responsible organisms have been isolated, and a preliminary account of them has been reported (Quastel, Scholefield & Stevenson, 1950). The present communication gives a more detailed account of these organisms and of the metabolism of pyruvic-oxime by one of them. Jensen (1951) has since reported the isolation of other organisms capable of producing nitrite from pyruvic-oxime.

METHODS

Preparation of materials. The preparation of the soil used in these experiments, the general methods and the estimations used in soil perfusion have already been fully described (Lees & Quastel, 1946; Quastel & Scholefield, 1949, 1951). A local soil was used throughout the present experimental work.

D-Arabinose-oxime was kindly supplied to us by Dr S. B. Baker, of this Institute, and formaldoxime, salicyl-

aldoxime, furil dioxime and benzoin oxime were Analar reagents. The oximes of acetone and acetoacetic ester were not isolated, a mixture of 1·1 moles of the ketone with 1·0 mole of hydroxylamine being used for the perfusion experiments.

The oximes of the sodium salts of pyruvic, oxalacetic, α-ketoglutaric and phenylpyruvic acids were prepared as follows: hydroxylamine hydrochloride (1·0 mole) and the free acid (1·1 moles) were mixed together and a little $NaHCO_3$ was added. The mixture was kept as a thick paste by the addition of small quantities of water as needed. Further $NaHCO_3$ was added until no more CO_2 was evolved and then sufficient water was added to bring the paste into solution at the boiling point in the presence of a slight excess of the alkali. On cooling, the solution deposited crystals of the sodium salts of the oximes of these α-ketoacids and it was in this form that they were used. The oxime of sodium pyruvate was generally recrystallized from boiling water to remove the brown colour normally associated with the pyruvic acid used. The samples of pyruvic-oxime used in this work contained no free pyruvate as estimated by yeast carboxylase and no free hydroxylamine as estimated by MnO_2 (Mann & Quastel, 1946). α-Ketobutyric acid oxime was prepared by Dr L. Martin of this Institute.

RESULTS

Perfusion of oximes through soil

The first oxime which was found to undergo metabolism in soil was pyruvic-oxime (Lees & Quastel, 1946; Quastel & Scholefield, 1949). It was later shown that oxaloacetic acid oxime suffers a similar breakdown (Quastel *et al.* 1950). To these must now be added phenylpyruvic acid oxime and α-ketoglutaric acid oxime, whilst salicylaldoxime, furil dioxime and arabinose oxime, do not yield appreciable quantities of nitrite in a period of 6 weeks. Chlorate is added in all cases to inhibit the further oxidation of nitrite (Lees & Quastel, 1945). Representative results are given in Table 1. It will be noted from this table that the oximes of oxaloacetic, phenylpyruvic and α-ketoglutaric acids do not inhibit markedly (if at all) the oxidation of pyruvic-oxime in soil. Similarily, D-arabinose oxime, whilst itself yielding little nitrite, in no way impedes the oxidation of pyruvic-oxime. Re-perfusion of arabinose oxime yielded no nitrite in 4 days. The stability of other sugar oximes in soil is now under consideration. Whilst only a dozen oximes have been studied to date, it seems likely that only those of the α-ketoacids are able to undergo nitrification in soil. A preliminary experiment has also shown that the hydrazones, unlike the oximes of these acids, are not nitrified.

Table 1. *Nitrite accumulation after perfusion of oximes through a soil*

(μg. Nitrite N accumulating per ml. perfusate after perfusion of various oximes (0·01 M) through soil in the presence of 0·001 M-sodium chlorate. 0·01 M-Oxime = 140 μg./ml.)

Time in hr.											
	Exp. 1	42		70		91		114		139	
	Exp. 2		43		66		91		115		138
	Exp. 3										300
0·01 M-Pyruvic-oxime	Exp. 1	48		113		113		110		114	
	Exp. 2		6		16		105		107		110
	Exp. 3										109
0·01 M-Arabinose oxime	Exp. 1	4		9		9		11		10	
0·01 M-Arabinose oxime + 0·01 M-pyruvic-oxime	Exp. 1	52		112		109		111		111	
0·01 M-α-Ketoglutaric acid oxime	Exp. 2		11		29		89		104		109
0·01 M-α-Ketoglutaric acid oxime + 0·01 M-pyruvic-oxime	Exp. 2		3		36		163		189		200
0·01 M-Oxaloacetic acid oxime	Exp. 3										98
0·01 M-Oxaloacetic acid oxime + 0·01 M-pyruvic-oxime	Exp. 3										203
0·01 M-Phenylpyruvic acid oxime	Exp. 1	1		22		40		58		62	
0·01 M-Phenylpyruvic acid oxime + 0·01 M-pyruvic-oxime	Exp. 1	57		132		150		154		153	
0·01 M-Salicylaldoxime	Exp. 3										3
0·01 M-Furil dioxime	Exp. 3										2

The oximes of acetone, formaldehyde, acetoacetic ester and benzoin are relatively inactive as nitrite formers in soil and do not inhibit the oxidation of pyruvic-oxime. It has also been found that disodium succinohydroxamate (prepared in this Institute by Dr L. Martin) does not inhibit this oxidation.

Certain antibiotics have been tested as possible inhibitors of pyruvic-oxime oxidation in soil. It has been found that penicillin and streptomycin are relatively inert. Thus, at a concentration of 0·01 % they produce only half a day's lag period with pyruvic-oxime as substrate. Chloromycetin produces a lag period varying from half a day at a concentration of 0·001 % to 6 days at 0·05 %.

The values for the lag periods are obtained by finding the difference between the times required to reach the asymptotic nitrite value in the presence and absence of the inhibitor. It is of interest to note that, at a concentration of 0·025%, chloromycetin entirely prevents oxidation of sodium nitrite in soil for a period of more than 10 days (Quastel & Scholefield, 1951).

Nitrourea at a concentration of 0·0033M completely inhibits nitrite oxidation for 16 days whilst having no effect on pyruvic-oxime oxidation (Quastel & Scholefield, 1951). Similarly, *p*-aminosalicylic acid produces a 1·5-day lag period in the oxidation of pyruvic-oxime in soil at a concentration of 0·0033M, but will produce a similar effect on the oxidation of ammonium ions at a concentration of 0·001M (Quastel & Scholefield, 1951).

At a concentration of 0·005% the only sulpha drug to have any effect on pyruvic-oxime oxidation is sulphadiazine, six others having relatively little effect.

Manometric experiments on washed suspensions of Achromobacter

As will be seen from the Addendum to this paper, four organisms have been isolated, three from soil and one from faeces, which can bring about the oxidation of pyruvic-oxime to nitrite. Manometric experiments have been carried out using suspensions of these organisms.

Pyruvic-oxime oxidase

The fact that the oxidation of pyruvic-oxime, which takes place in the presence of these organisms, is controlled by a thermolabile enzyme was established by showing that immersion of the organisms in a boiling-water bath for 1 min. completely eliminated their respiratory activity in the presence of pyruvic-oxime. Furthermore, the presence of M/300 sodium cyanide inhibited the oxygen uptake by over 70% and M/300 sodium azide by over 90%. The enzyme controlling the oxidation was termed pyruvic-oxime oxidase (Quastel *et al.* 1950).

Whilst it was quite definite that the three soil organisms and the one isolated from faeces could all metabolize pyruvic-oxime and produce nitrite from this source, their relative activities differed considerably. Suspensions of each organism were therefore prepared and the rates of oxygen uptake in air were measured using potassium hydroxide in the centre wells of the Warburg vessels. In each case the turbidities were made the same, as judged with the Fisher colorimeter using Filter B 525, and in each case the endogenous metabolic rate was also estimated. Fig. 1 shows that both *Achromobacter* sp. (1) and the faecal organism actively metabolize pyruvic-oxime. The other species of *Achromobacter* and the species of *Corynebacterium*, on the other hand, showed only a low rate of oxidation of pyruvic-oxime under these conditions. In all the experimental work discussed in this paper, therefore, the *Achromobacter* sp. (1) has been used. The organisms were always washed twice with distilled water and suspended in distilled water. The suspension contained the growth from one 3-day-old plate per ml. Approximately 5 mg. dry weight washed organisms was used per vessel in all experiments.

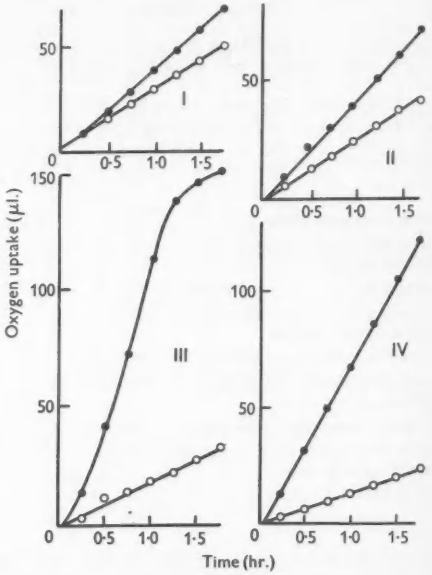

Fig. 1. Comparison of the abilities of four organisms to oxidize pyruvic-oxime. Contents of flasks: 1·0 ml. washed suspension of organisms (5 mg. dry wt.), 1·0 ml. 0·1M-phosphate buffer, pH 7·2; 0·2 ml. 0·02M-pyruvic-oxime; water to total vol. 3·2 ml. Air 37°. KOH in centre wells. (I) *Corynebacterium* sp. from soil; (II) *Achromobacter* sp. (2) from soil; (III) *Achromobacter* sp. (1) from soil; (IV) *Achromobacter* sp. (3) from faeces; ○, no substrate added; ●, 0·2 ml. 0·02M-pyruvic-oxime present.

In view of our previous studies concerning the effects of hydrogen-ion concentration on the production of nitrite in soils (Quastel & Scholefield, 1949, 1951) it seemed of interest to investigate the effects of changes of pH on the oxidation of pyruvic-oxime. It will be seen from Fig. 2 that in the range of pH 6–9 there is no appreciable variation in the rate of O_2 consumption, but that a shift of 0·5–1·0 pH unit outside this range causes a very sharp drop in activity. In all our experiments on the production of nitrite from pyruvic-oxime in soil, therefore, pH may be eliminated as a factor in-

fluencing metabolic activity since the buffering power of soil is great enough to maintain conditions well within the region of pH 6–9.

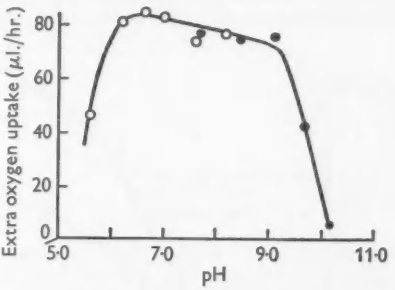

Fig. 2. The variation of activity of pyruvic-oxime oxidation with pH. ○, phosphate buffer (0·066 M); ●, borate buffer (final concentration of H_3BO_3, and KCl 0·05 M; pH adjusted with NaOH). *For experiment*: 2·0 ml. buffer solution; 1·0 ml. bacterial suspension; 0·2 ml. 0·25 M-pyruvic-oxime or 0·2 ml. water tipped.

Oxygen consumption during the oxidative metabolism of pyruvic-oxime

It was of interest to discover the number of molecules of O_2 consumed by the organism during its metabolism of one molecule of pyruvic-oxime and whether the ratio of O_2 consumed to pyruvic-oxime used remained constant during the course of an experiment. It has been found convenient to estimate the amount of nitrite formed, as this is a measure of the metabolism of pyruvic-oxime.

Table 2. *Comparison of oxygen uptake and nitrite production during metabolism of pyruvic-oxime*

(Details given in text.)

Time (min.)	Extra oxygen uptake (i) (μl.)	Nitrite produced (ii) (1 g.mol. = 22·4 l.) (μl. equivalent)	Ratio: (i)/(ii)
15	4·1	2·24	1·8
30	7·4	4·62	1·6
45	13·6	7·54	1·8
60	21·8	—	—
75	30·0	15·3	1·95
90	35·7	20·2	1·8
105	55·4	31·2	1·9
120	71·0	37·6	1·9
135	79·5	41·5	1·9
		Mean	1·82

The results of a typical experiment are given in Table 2. Several manometer vessels were set up containing 1·0 ml. bacterial suspension of the standard organisms, 1·0 ml. 0·1 M-phosphate buffer, pH 7·2, and 1·0 ml. water; and into each of them was tipped 0·2 ml. 0·2 M-pyruvic-oxime. Every 15 min. one vessel was removed and the nitrite content of the medium was estimated colorimetrically. From the results given in the above table it will be seen that 1·82 molecules of O_2 are consumed per molecule of sodium nitrite produced. The value of this figure indicates that the molecule of pyruvic-oxime is not completely oxidized by these organisms under our experimental conditions. The theoretical value of the ratio in question for complete oxidation to CO_2, water and nitrite would be 3·5. Furthermore, it is obvious that the ratio of nitrite produced to O_2 consumed is constant within experimental error during the entire experiment.

A likely mode of breakdown of pyruvic-oxime is given by the following scheme:

$$CH_3.C{:}(NOH)COOH + O_2 \rightarrow CH_3.CO.COOH + HNO_2$$
$$\downarrow \tfrac{1}{2}O_2$$
$$CH_3.COOH + CO_2$$

Such a scheme involves the necessary intermediate formation of pyruvic acid. Experiments were therefore carried out to discover whether the presence of semicarbazide, by its binding of intermediary pyruvic acid, would influence the rate of O_2 uptake of *Achromobacter* in the presence of pyruvic-oxime. The results showed that semicarbazide (0·05 M) neither altered the rate of O_2 uptake nor, within experimental error, the total O_2 uptake.

The oxygen uptake due to the addition of other substances

(*a*) *Nitrogen-containing molecules.* The metabolism of a number of substrates by *Achromobacter* sp. (1) has been studied with the intention of discovering which compounds are possible intermediates in the metabolism of pyruvic-oxime. Thus one pathway of metabolism of pyruvic-oxime might be via alanine by reduction of the oxime to this amino-acid. That this is not the case, however, is shown by the facts: (i) that the metabolism of the oxime in soil does not proceed via ammonia, whereas that of alanine does (Quastel & Scholefield, 1949); (ii) that alanine on incubation with *Achromobacter* sp. (1) produces no nitrite although it is quite readily oxidized. Another possible intermediate is hydroxylamine, but previous evidence has already rendered this possibility unlikely. Again 0·0001 M-hydroxylamine does not produce nitrite on perfusion in soils that readily oxidize pyruvic-oxime.

Since it is clear that neither ammonia nor hydroxylamine is likely to be an intermediate in the breakdown of pyruvic-oxime and since the ratio of O_2 uptake to the formation of nitrite is constant from the start of the experiment, it is reasonable to deduce that the nitrite is formed by an initial direct oxidation of the oxime.

(*b*) *Oxidation of possible intermediates in the metabolism of pyruvic-oxime.* Investigations were made of the rates of O_2 uptake by a washed suspension of

Achromobacter sp. (1), in the presence of a variety of substances which might be possible intermediates in pyruvic-oxime metabolism. Sodium lactate and sodium pyruvate were found to be oxidized by the organism without any initial time lag; the rates of O_2 uptake were not constant but tended to increase with time, a phenomenon also shown by pyruvic-oxime. Sodium propionate was also oxidized, but the onset of oxidation is preceded by an initial time lag. Constant rates of O_2 uptake have been obtained with acetate, but usually even this substrate tends to give an increasing rate of O_2 uptake with time. Typical results for pyruvic-oxime and sodium pyruvate oxidation are given in Figs. 3 and 4. The results shown in Table 3 were obtained with a freshly harvested suspension of organisms that had been subcultured from a stock stored for 6 months at room temperature after lyophilization. Enzymic activity with pyruvic-oxime as substrate remained high but the extent of oxidation was somewhat lowered.

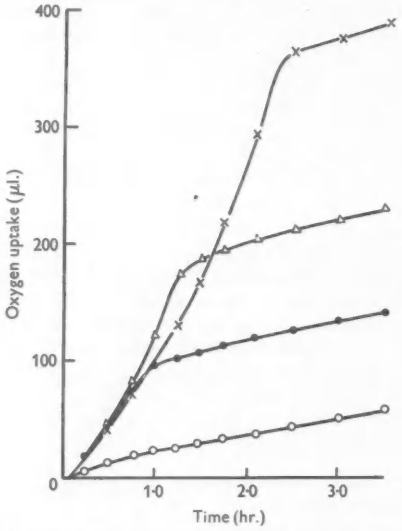

Fig. 3. The effect of pyruvic-oxime concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but with varying amounts of pyruvic-oxime. ○, no pyruvic-oxime present; ●, 0·1 ml. 0·02 M-pyruvic-oxime; △, 0·2 ml. 0·02 M-pyruvic-oxime; ×, 0·4 ml. 0·02 M-pyruvic-oxime.

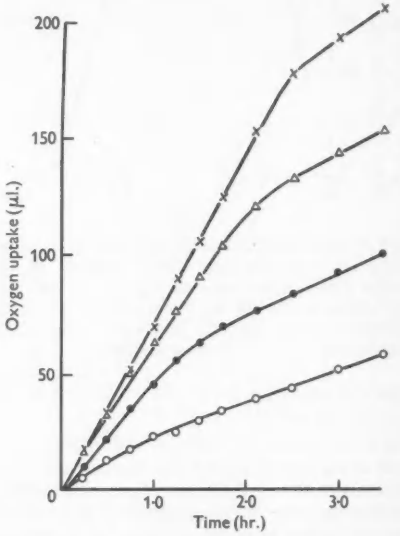

Fig. 4. The effect of sodium pyruvate concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but no pyruvic-oxime present. ○, no sodium pyruvate present; ●, 0·1 ml. 0·02 M-sodium pyruvate; △, 0·2 ml. 0·02 M-sodium pyruvate; ×, 0·4 ml. 0·02 M-sodium pyruvate.

In this experiment the nitrite yield was 75% of the theoretical. It would therefore be expected that 1·50 atoms of O would be taken up per molecule of pyruvic-oxime added, on the assumption that oxidation proceeds initially to pyruvate and nitrite. The further oxidation of the pyruvate produced would yield another 1·79 × 0·75, or 1·34, atoms of O per molecule of pyruvic-oxime (Table 3). Thus the oxidation of the oxime by these organisms would require 1·50 + 1·34, or 2·84, atoms of O per molecule and this is in agreement with the observed value of 2·81. This observation conforms with the view that pyruvate is an essential intermediate in the oxidation of pyruvic-oxime.

Table 3. *Atoms of oxygen taken up per molecule of substrate, after lyophilization and storage of* Achromobacter *sp.* (1)

Pyruvic-oxime	2·81
Sodium pyruvate	1·79
Sodium acetate	2·24

It will be noted from the results given in Table 3 that the O_2 uptake per molecule of substrate is of the same order with sodium acetate as with sodium pyruvate. This result we consider explicable on the basis that part of the pyruvate (about 40%) is assimilated in synthetic processes in the organism, the remainder undergoing further oxidation.

(*c*) *The metabolism of other substrates.* When the sodium salts of succinic, fumaric and malonic acids were tested as substrates, the curves shown in Fig. 5

Table 4. *The effect of various oximes on the metabolism of pyruvic-oxime*

(Present in all flasks: 1·0 ml. suspension; 1·0 ml. 0·1 M-phosphate buffer, pH 7·2; 0·2 ml. 0·02 M-pyruvic-oxime; 0·2 ml. 0·02 M-inhibitor and water to total vol. of 3·2 ml. Air. 37°. KOH in centre wells.)

Inhibitor	Oxygen uptake in 45 min. (μl.)	Correction for O_2 uptake in the absence of pyruvic-oxime and in the presence of the inhibitor (μl. O_2)	Percentage inhibition of rate of O_2 uptake
None	120	19	0
Phenylpyruvic acid oxime	110	43	33
D-Arabinose oxime	80	25	46
α-Ketoglutaric acid oxime	98	30	32

were obtained. They indicate that malonate is not metabolized by this organism, succinate after a short lag period, and fumarate but feebly.

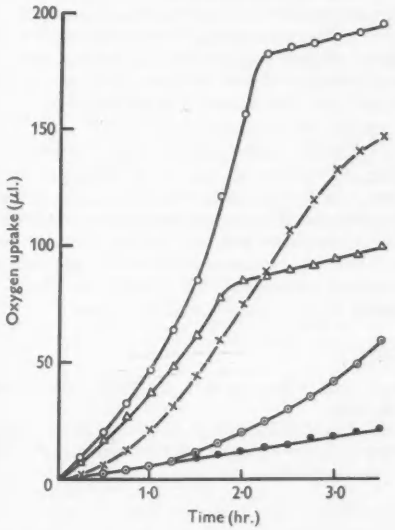

Fig. 5. The oxidation of various carboxylic acids by *Achromobacter* sp. (1) compared with that of pyruvic-oxime. Contents of flasks as in Fig. 1 with the following substrates replacing pyruvic-oxime. ●, water or sodium malonate (0·2 ml. 0·02 M); ⊙, sodium fumarate (0·2 ml. 0·02 M); ×, sodium succinate (0·2 ml. 0·02 M); △, sodium pyruvate (0·2 ml. 0·02 M); ○, pyruvic-oxime (0·2 ml. 0·02 M).

(*d*) *The effects of other oximes.* The effects of pure samples of the oximes of α-ketobutyric acid, phenylpyruvic acid, α-ketoglutaric acid and arabinose on pyruvic-oxime oxidation were investigated (Quastel *et al.* 1950). The results are shown in Table 4 and indicate inhibitory effects of three of these oximes.

Phenylpyruvic acid oxime solution usually shows no free nitrite, and yet after 2 hr. incubation with the organism this substance consistently gave 5–10% of its total nitrogen content as free nitrite. Some increase in O_2 uptake is obtained (Table 4), but this is also seen to some extent with the other two oximes which yield no nitrite. This activity is so feeble, however, that it has been classified as approximately zero in considering enzyme substrate specificities. The other two oximes have never yielded any nitrite with this organism.

Table 5. *Metabolism of oximes by* Achromobacter *sp.* (1)

(Conditions as in Table 4)

Substrate added (μmoles)		Oxygen uptake in 60 min. (μl.)	Nitrite formed finally (μmoles)
Pyruvic-oxime	α-Ketobutyric acid oxime		
0	0	30	0
10	0	225	7·39
0	10	117	9·36
10	10	184	16·76

It is evident, therefore, that whilst the oxidation of various oximes to nitrite in soil is confined to those of the α-ketoacids, the ability of isolated organisms to oxidize the oximes is more restricted. Thus *Achromobacter* sp. (1) can metabolize pyruvic-oxime but not (or only very feebly) the oximes of the α-ketoacids mentioned above, although some of these are quite readily metabolized on perfusion through soil. One other oxime is, however, metabolized by this organism, i.e. the oxime of α-keto-butyric acid. Evidence for this is presented in Table 5. Both oximes are readily oxidized, but admixture causes the rate of O_2 uptake to fall between the values given by each oxime alone. This would indicate that the two substrates compete for a single enzyme system. Nitrite formation at the completion of the experiment accounts for 74% of the added pyruvic-oxime nitrogen and 94% of the added α-ketobutyric acid oxime nitrogen. In a mixture of the two oximes the nitrite yield is ultimately completely additive.

No period of adaptation seems to be necessary before the oximes are metabolized, and in no manometric experiment has any evidence been obtained which would indicate that *Achromobacter* sp. (1) may become adapted to oxidize any oxime other than those of the α-keto fatty acids.

SUMMARY

1. Oximes of the α-ketoacids (pyruvic, phenylpyruvic, oxaloacetic and α-ketoglutaric) are readily nitrified in soil.

2. The oximes of salicylaldehyde, furil, arabinose, acetone, formaldehyde, acetoacetic ester, and benzoin are inert in soil as nitrite-formers and do not inhibit nitrification of pyruvic-oxime in soil.

3. Among six sulphanilamide derivatives tested, sulphadiazine (0·005%) has the most marked inhibitory action on nitrite formation from pyruvic-oxime in soil. Chloromycetin (1 in 40000) is highly inhibitory, but penicillin and streptomycin are relatively inert under soil conditions. *p*-Aminosalicylic acid has a small inhibitory action, but nitrourea (0·0033 M), which completely inhibits nitrite oxidation in soil, has no inhibitory effect on nitrite formation from pyruvic-oxime.

4. Details are given of the isolation and characterization of organisms in soil and in faeces capable of oxidizing pyruvic-oxime. Three species of organisms, attacking pyruvic-oxime, have been isolated from soil. Two of the species fall into the genus *Achromobacter*; the third is identified as a *Corynebacterium*.

5. Among twenty-two stock cultures of bacteria tested for their abilities to proliferate on a pyruvic-oxime medium, a few gave sparse growth and none formed nitrite.

6. Using washed suspensions of *Achromobacter* sp. (1) it is found that during the oxidation of pyruvic-oxime, the ratio of nitrite production to oxygen uptake remains constant.

7. The results of perfusion and manometric experiments indicate that alanine is not an intermediate in pyruvic-oxime oxidation. Hydroxylamine and ammonia are also unlikely to be intermediates.

8. Suspensions of resting *Achromobacter* sp. (1) oxidize acetate and pyruvate readily, succinate after a short lag period and fumarate very slowly. Malonate is not attacked.

9. The evidence would indicate that pyruvic-oxime is oxidized to pyruvate and nitrite, the pyruvate then undergoing further breakdown. The enzyme system responsible for the oxidation of pyruvic-oxime is termed pyruvic-oxime oxidase. Sodium azide and sodium cyanide markedly inhibit the activity of pyruvic-oxime oxidase.

10. Pyruvic-oxime oxidation by suspensions of resting *Achromobacter* sp. (1) is inhibited by the oximes of α-ketoglutaric acid, phenylpyruvic acid and arabinose. These oximes are apparently either feebly or not attacked by pyruvic-oxime oxidase. The oxime of α-ketobutyric acid is also oxidized by washed suspensions of *Achromobacter* sp. (1), probably by the pyruvic-oxime oxidase.

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ADDENDUM

The Isolation of Bacteria from Soil Perfused with Pyruvic Acid Oxime

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Approximately 2 g. of perfused soil 'crumbs' were brought into a finely particulate suspension in 6 ml. of sterile distilled water by gently grinding in a mortar. Larger soil particles were allowed to settle out and the supernatant fluid which contained the smaller particles in suspension was cultured. The culture medium, which contained pyruvic-oxime as the only source of assimilable carbon and nitrogen, was prepared as follows: K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; NaCl, 0·2 g.; $MnSO_4.4H_2O$, trace; $FeCl_2.6H_2O$, trace; Granular Agar (Difco), 15 g.; pH adjusted to 7·0 with NaOH. Dispensed in 100 ml. lots and autoclaved at 120° for 20 min. 0·25 M-Pyruvic-oxime (10 ml.) rendered sterile by Seitz filtration was added to each 100 ml. lot of molten agar-salt solution base and plates were poured.

An inoculum of 0·1 ml. of the soil suspension per plate, spread evenly over the surface of the agar, provided cultures in which colonies were, for the most part, discrete. Plates were incubated at 25 and 37°. Despite the fact that perfusion had been carried out at 21° optimal growth was obtained at 37°. There was no selective effect of incubation temperature, plates incubated at 25 and 37° showing the same flora. Incubation for 72 hr. was required to bring out characters by which various colony types might be distinguished one from the other. No new forms appeared on continued incubation beyond this line.

A number of picks of each colony type were grown in pure culture on pyruvic-oxime agar in order to test their ability to attack pyruvic-oxime with the liberation of free nitrite. The test consisted in removing a small square of the agar bearing the growth (0·5 sq.cm.) which was dropped into Ilosvay reagents in a tube. All picks gave strong tests for nitrite. Control squares of sterile pyruvic-oxime agar incubated for the same period of time showed no trace of nitrite. The various picks upon taxonomic study were resolved into three distinct species. It is of interest to note that all of the organisms isolated from the soil on the pyruvic-oxime medium were capable of oxidizing pyruvic-oxime with the production of nitrite. This would tend to suggest a process of selection or adaptation or perhaps a combination of both phenomena during perfusion.

Two of the species isolated from the perfused soil fall into the genus *Achromobacter*; the third has been identified as a *Corynebacterium*. A third species of the genus *Achromobacter* capable of oxidizing pyruvic-oxime with the formation of nitrite has been isolated from a sample of faeces. None of the species which were isolated conforms with species described under the genera *Achromobacterium* and *Corynebacterium* in Bergey's Manual (1948). Due to the unsettled state of classification of species within these genera the organisms isolated have not been given specific designations.

The following are the characteristics of the various species.

Achromobacter sp. (1)

From perfused soil.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0·5 by 1–3 μ. No endospores. Not encapsulated. Motile, possessing peritrichous flagella. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2–3 mm. in diameter, low convex, circular with entire edges, grey, smooth and glistening, translucent, mucoid. Growth in peptone broth turbid with pellicle and sediment, aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. Cannot use salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (2)

From perfused soil.

Morphology. Rods with rounded ends occurring singly and in pairs. Coccoid and oval forms 0·5–1 by 1–3 μ. No endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, grey, smooth and glistening, translucent. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (3)

A third species of *Achromobacter* capable of oxidizing pyruvic-oxime with nitrite production was isolated from faeces and characterized as follows.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0·5 by 1–3 μ. No endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2–3 mm. in diameter, low convex, circular with entire edges, grey to faint cream colour (no pigment on colourless media), smooth and glistening, translucent. Non-mucoid. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates very slowly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. Acid from dextrose and galactose. No acid from arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

The essential points of difference upon which the separation of the foregoing organisms as distinct species is based are outlined in Table 1.

Table 1. *Distinguishing features of three species of* Achromobacter *which oxidize pyruvic-oxime*

Test	*Achromobacterium* sp. (1)	*Achromobacterium* sp. (2)	*Achromobacterium* sp. (3)
Motility	+	–	–
Nitrate reduction	Rapid	Rapid	Very slow
Acid from dextrose	–	–	+
Acid from galactose	–	–	+
Use of salts of citric acid as sole source of carbon	–	+	+

Corynebacterium sp.

From perfused soil.

Morphology. Slightly curved rods, 0·5–1 by 2–4 μ. in young culture. 1 by 6–8 μ. in older culture. Palisading and angular arrangement. Pleomorphic on ageing, with club forms. No endospores. Not encapsulated. Non-motile.

Uniformly Gram-positive in young culture tending to variability in older culture with Gram-negative cytoplasm beaded with Gram-positive granules. Not acid-fast.

Cultural characteristics. Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, white, smooth non-glistening surface. Growth in peptone broth: granular sediment with little general turbidity. Aerobic. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates slowly reduced to nitrites. H_2S not produced. Indole not formed. Cellulose not digested. Starch not hydrolysed. Acid from levulose. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Unless otherwise indicated, *Achromobacter* sp. (1) has been used for all the manometric studies.

In order to determine whether or not species of bacteria other than those isolated from the perfused soil might attack pyruvic-oxime with the production of nitrite, a series of laboratory stock cultures which had been maintained on peptone agar were transferred to pyruvic-oxime agar. The results are outlined in Table 2. It is seen that none of a variety of species held in stock possessed, initially at least, the ability to produce nitrite from pyruvic-oxime. Indeed many of the species tested failed to grow on this medium. Growth where it occurred was in all instances rather sparse requiring from 3 days to 1 week of incubation, depending upon the species. The finding that certain heterotrophic organisms may oxidize pyruvic-oxime to nitrite has been confirmed by the recent work of Jensen (1951).

It was felt that adaptation might be a factor in determining the ability of an organism to attack pyruvic-oxime. In order to test this, four selected species, *Achromobacter hartlebii*, *Achromobacter perolens*, *Agrobacterium radiobacter* and *Bacterium globiforme*, were carried through six successive transfers on pyruvic-oxime agar. On the sixth transfer the two species of *Achromobacter* produced very small amounts of nitrite. *Agrobacterium radiobacter* and *Bacterium globiforme* failed to produce nitrite. It is of interest to note that the two species which gained, presumably by adaptation, the ability to attack the substrate with the liberation of free nitrite were both members of the same genus as the three active species isolated from perfused soil and faeces.

Table 2. *The ability of various stock cultures of bacteria to grow on pyruvic-oxime agar and to produce nitrite from pyruvic-oxime*

Organism	Growth	Nitrite
Achromobacter hartlebii	+	–
Achromobacter perolens	+	–
Achromobacter sp. (isolated from fish)	+	–
Alcaligenes faecalis	+	–
Agrobacterium radiobacter	+	–
Chromobacterium violaceum	–	–
Pseudomonas aeruginosa (2 strains)	+	–
Escherichia coli	–	–
Aerobacter aerogenes	+	–
Klebsiella pneumoniae	+	–
Serratia marcescens	+	–
Proteus vulgaris	–	–
Micrococcus ureae	–	–
Micrococcus pyogenes var. *aureus*	–	–
Micrococcus epidermidus	–	–
Micrococcus agilis	–	–
Sarcina lutea	–	–
Corynebacterium segmentosum	–	–
Bacterium globiforme	+	–
Bacillus mesentericus	–	–
Bacillus cereus	–	–
Bacillus megatherium	–	–

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The Isolation of Androst-16-en-3α-ol from Women's Urine

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The musk-smelling androst-16-en-3α-ol, first isolated from swine testes by Prelog & Ruzicka (1944), was obtained from a glucuronide fraction from men's urine by Brooksbank & Haslewood (1949, 1950) and from the urine of women with adrenal cortical tumours by Mason & Schneider (1950). When Brooksbank & Haslewood (1950) tried to obtain this steroid from the urine of women not showing any endocrine abnormality, they were able to isolate from the musk-smelling fraction only a small amount of a substance melting at about 160°, and therefore different from the androstenol. It has seemed of interest to us to repeat this work in order to settle, if possible, the question of the nature of the musk-smelling constituent of (hydrolysed) urine from normal women.

RESULTS

The work described below shows that the urine examined contained androst-16-en-3α-ol (approximately 0·2–0·3 mg./l.), as well as pregnane-3α:20α-diol (in a concentration of the order of 0·4 mg./l.); these substances were almost certainly present as glucuronides. The previously described substance of melting point about 160° was not again encountered.

DISCUSSION

The urine used was collected from women in whom there was no evidence of endocrine abnormality, and it probably consisted almost entirely of 'follicular' urine, i.e. excreted in the menstrual cycle before the formation of functioning corpora lutea. It is clear, therefore, that in man, the androstenol is truly characteristic of female, as well as of male, urine. It may be of adrenal origin, as suggested by Mason & Schneider (1950) for a female case of adrenal cortical neoplasm. There is no definite evidence to suggest that the testis is involved in its formation by the male, although it was our impression that rather greater amounts were excreted by men than by women. The amount of pregnanediol isolated was about what might have been expected in 'follicular' urine.

METHODS

In general the methods of isolation of Brooksbank & Haslewood (1950) were closely followed.

'Pregnanediol-like glucuronide' (PLG) was estimated as previously described, and the results expressed as pure sodium pregnanediolglucuronidate (NaPG). Melting points are uncorrected. Light petroleum was A.R., b.p. 40–60°. Al_2O_3, supplied by Hopkin and Williams Ltd., was neutralized as described by Shoppee (1949).

Isolation and purification of PLG. Urine (191 l.) was collected from healthy women and from patients (in surgical wards) not suffering from endocrine diseases, cancer, kidney or bladder disturbances. All subjects menstruated normally; they were instructed to collect urine only during the first 14 days after menstrual bleeding had ceased. Urine was preserved with thymol-salicylic acid or butanol. It was extracted with butanol and the PLG entrained as described by Brooksbank & Haslewood (1950) in a manner corresponding to the 'concentrated' conditions of the original small-scale estimation (Bisset, Brooksbank & Haslewood, 1948). The total yield of PLG recovered from barium phosphate precipitates was 1·629 g., which was assayed as approx. 50% pure glucuronide (as NaPG). Of the 1·629 g., 96 mg. was used in experiments, kindly carried out by Dr Mary Barber, on bacterial hydrolysis; it was found that this was unsatisfactory, as the extracts apparently contained an inhibitor of glucuronidase activity. An unsuccessful attempt to remove the glucuronidase inhibitor was made by dissolving the remaining PLG (1532 mg. =789 mg. NaPG) in *n*-butanol/methanol (2:1 v/v) and eluting it from Al_2O_3 (10 g.) in a swiftly moving column (2 × 3 cm.). The original solvent eluted 283 mg. (27% NaPG), methanol (300 ml.) gave 956 mg. (39% NaPG) and further methanol (approx. 1 l.) gave 147 mg. (48% NaPG). The total solid recovered thus weighed 1·386 g.; it was equivalent to 517 mg. NaPG and hence 789–517 = 272 mg. (34%) of glucuronide (as NaPG) were lost on the column.

Enzymic hydrolysis of PLG. The PLG (517 mg. as NaPG) recovered from Al_2O_3 (above) in 0·1 M-citrate buffer (770 ml., pH 5·2, Kerr, Graham & Levvy, 1948), was treated with 18 200 units of purified calf-spleen glucuronidase (prepared according to instructions kindly supplied by Dr G. T. Mills of the University of Glasgow) dissolved in water (90 ml.). The mixture, together with about 5 ml. $CHCl_3$, was incubated at 37°. Commercial deoxyribonucleic acid, to a concentration of about 0·3% (w/v), was added after 2 days as a glucuronidase accelerator (Bernfeld & Fishman, 1950) and incubation was continued for a further 2·5 days. The mixture was then diluted with water to about 1 l. and extracted continuously with ether for about 16 hr.; the aqueous portion was finally shaken with two lots of about 200 ml. each of fresh ether. The combined ether extracts were washed with water, dilute Na_2CO_3 solution, water, dried (Na_2SO_4) and evaporated. The residue weighed 247 mg.; it was partially crystalline and had a strong musk-like smell. If this residue had been pregnanediol, it would correspond to about 401 mg. of NaPG; hence about 517–401 = 116 mg. of glucuronide (as NaPG) remained unhydrolysed. Hence, the approximate volume of urine from which the above 247 mg. of hydrolysed material was obtained corresponded to

$$\frac{1532}{1629} \times \frac{401}{789} \times 191 = 91 \text{ l.}$$

Isolation of steroids. The above hydrolysed ether-soluble neutral material (247 mg.) was left for a few days at 0–5° with acetone (about 10 ml.). The separated solid was collected and washed with a little cold acetone. The insoluble material (58 mg.) was crystallized from ethanol/aqueous NaOH and gave pregnane-3α:20α-diol (30 mg., m.p. 228–232°, not depressed by authentic material).

Evaporation of the above acetone liquors left a strongly smelling brown gum (189 mg.) which was purified by chromatography as shown in Table 1.

Fraction 4 (75·2 mg.) from column 1 was crystallized from ethanol/aqueous NaOH and gave pregnane-3α:20α-diol (5 mg., m.p. 224–227° not depressed by an authentic sample). Hence, the total weight of purified pregnanediol isolated was about 35 mg., i.e. about 0·4 mg./l. of urine.

Fractions 5 (5·8 mg.) and 6 (2·0 mg.) from column 2 were purified by cooling with a little light petroleum in solid CO_2-acetone mixture, decanting and washing the crystalline residue with a little light petroleum similarly cooled. The residues were recrystallized from aqueous acetone, as also was the residue left on evaporation of the light petroleum liquors. The three samples (none of which weighed more than about 1 mg.) thus obtained had melting points as follows: from fraction 5, m.p. 140–142°; from fraction 6, m.p. 136–138°; from the combined light petroleum liquors, m.p. 142–143°. None of these melting points was depressed by mixture with authentic androst-16-en-3α-ol. The last sample (m.p. 142–143°) was sent to Dr K. Dobriner, of the Sloan-Kettering Institute for Cancer Research, New York, for an infrared spectral examination. Dr Dobriner reported that the absorption taken in the 'finger-print' region (900–1200 $cm.^{-1}$) of our sample was identical with that of

Table 1. *Chromatography of hydrolysed ether-soluble neutral material from women's 'follicular' urine*

Fraction no.	Eluted with: Vol. (ml.)	Eluted with: Solvent	Eluate: Wt. (mg.)	Eluate: Appearance
		Column 1. Hydrolysed material (189 mg.) on Al_2O_3 (2 g.)		
1	40	50% (v/v) Benzene/light petroleum	73·1	Gum
2	40	50% (v/v) Benzene/light petroleum	9·3	Gum
3	40	Benzene	7·8	Gum
4	40	Ether	78·2	Crystalline
5	40	Acetone	10·0	Gum
6	40	Ethanol	10·0	Gum
		Total	188·4	
		Column 2. Fraction 1 (73·1 mg.) of column 1 on Al_2O_3 (1 g.) in a column (5 × 0·5 cm.)		
1–3	10	Light petroleum	20·4	Colourless oil, slight smell
4	5	Light petroleum	18·6	Reddish oil, musk smell
5	10	Light petroleum	5·8	Crystalline, musk smell
6	10	Light petroleum	2·0	Crystalline, musk smell
7	20	Light petroleum	4·3	Partially crystalline, musk smell
8	10	50% (v/v) Benzene/light petroleum	7·4	Brown gum, smell
9	20	Benzene	12·6	Gum
		Total	71·1	

androst-16-en-3α-ol. Less pure samples of the androstenol were obtained from fractions 7 and 8 of column 2: more of this substance might have been present in fraction 4 from this column. The previously purified substance of m.p. 160–161° (Brooksbank & Haslewood, 1950) was not detected.

SUMMARY

1. Enzymic hydrolysis of a partially purified 'pregnanediol-like glucuronide' fraction from the urine of women in the 'follicular' phase of the menstrual cycle gave a mixture from which was isolated androst-16-en-3α-ol and pregnane-3α:20α-diol.

2. The amount of the latter substance obtained indicated an original concentration of the order of 0·4 mg./l. of the urine, whilst the androstenol might have been present in amounts of 0·2–0·3 mg./l. The musk-smelling androst-16-en-3α-ol is thus truly characteristic of the (hydrolysed) urine of men and women.

The authors thank Dr K. Dobriner for the infrared spectral identification, also Dr Mary Barber for several experiments on bacterial hydrolysis and Dr F. T. G. Prunty for advice. Without the generous co-operation of Staff and patients at St Thomas's Hospital this work could not have been undertaken.

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